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SIMON FLEXNER, M.D. PEYTON ROUS, M.D.

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SURVIVAL AND GROWTH OF FIBROBLASTS IN VITRO.

By ALEXIS CARREL, M.D., and ALBERT H. EBELING, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, June 5, 1923.)

Although the nature of the nitrogenous compounds used by the various tissues in the process of growth is not known, it may be assumed that all cells do not have the same food requirements. Some may receive the nitrogen necessary for the synthesis of new protoplasm from certain amino-acids, peptides, or proteins, while others may require more complex compounds elaborated by other cells. The fibroblasts which, when cultivated in blood serum,[1] multiply temporarily but never increase in mass, proliferate indefinitely as soon as a small amount of embryonic tissue juice[2] is added to the medium. It appears then that the survival or growth of the tissues depends upon the nature of the nitrogenous compounds contained in the medium. It is of interest to know which substances determine the building up of new cells by fibroblasts, and which cannot be utilized for multiplication. Therefore, a study was made of the effect on the proliferation of fibroblasts of egg white, pure egg albumin, egg yolk, and chicken bouillon.

EXPERIMENTAL.

The nutritive value of egg white, egg albumin prepared by the methods of Sörensen and Hopkins, egg yolk, and chicken bouillon, was tested on chicken fibroblasts which were obtained from an 11 year old strain. In most of the experiments, the method of washing and transferring the tissues from medium to medium was used. The control medium was composed of fibrinogen suspension, Tyrode solution with traces of egg yolk, and embryonic tissue juice. In the experiments, the substance to be tested was substituted for an equal amount of Tyrode solution.

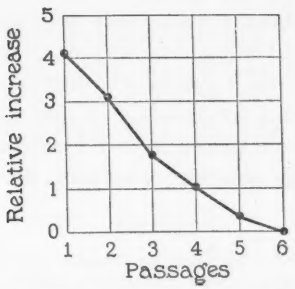
[1] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 317; 1922, xxxvi, 399.

[2] Carrel, A., *J. Exp. Med.*, 1912, xv, 516. Ebeling, A. H., *J. Exp. Med.*, 1922, xxxv, 755.

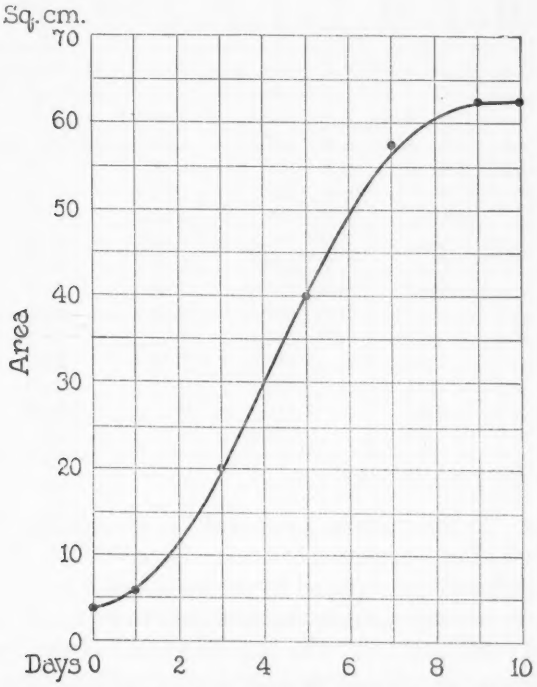
Every 48 hours, the growth was measured, and the tissues were washed and transferred into a fresh medium. The same operation was repeated until the tissues died or until their rate of growth became uniform. The effects of Tyrode solution and of the substance under investigation were compared from the point of view of the amount of tissue produced at each passage, and of the duration of life of the cultures. In other experiments, the tissues were studied in the process of uninterrupted growth in a medium which was renewed every 2 or 3 days. The fibroblasts were cultivated in D-5 flasks and imbedded in a coagulum made of 0.5 cc. of plasma and 1.5 cc. of Tyrode solution containing 5 per cent embryo juice. The substance to be tested was poured on the surface of the coagulum. The area of the tissues was measured every 48 hours. The curve expressing the growth of the tissues in the experimental medium was compared to that of the same tissue when the fluid part of the medium was composed of Tyrode solution.

1. Action of Tyrode Solution on Pure Cultures of Fibroblasts.—Rectangular fragments of an 11 year old strain of chicken fibroblasts were placed in a medium composed of chicken fibrinogen 25 per cent, doubly concentrated Tyrode solution 25 per cent, Tyrode solution containing traces of egg yolk and tissue extract 25 per cent, and Tyrode solution 25 per cent.[3] The migration of the fibroblasts began after about 2 hours incubation, and the cells spread rapidly into the coagulum. After 48 hours incubation, the area covered by the new tissue was always large, although the amount of extract contained in the medium was far too small to produce any stimulating effect. The tissues were extirpated from the clot, washed, and transferred to a fresh medium. After each passage the cells proliferated rapidly but, in spite of this apparent activity, the mass of the tissue slowly decreased. A visible, progressive thinning of the central portion of the culture was apparent even on macroscopic observation of the mother fragment. The cells multiplied by no means as rapidly as they migrated. After each passage the area of the fragment not only lessened but the thickness also decreased. As the medium contained no nitrogenous compounds which could be used by the cells, protoplasm could not be synthetized, and the loss of tissue which took place at each passage brought about a rapid decrease in its mass. Under the conditions of the experiment, death occurred generally after from five to six passages, that is, from 8 to 12 days (Text-fig. 1).

[3] Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiii, 641.



TEXT-FIG. 1. Action of Tyrode solution on pure cultures of fibroblasts. The relative increase of pure cultures of fibroblasts during one passage, that is, 48 hours, is plotted in ordinates, and the number of passages in abscissæ.



TEXT-FIG. 2. Action of Tyrode solution on pure cultures of fibroblasts in a condition of uninterrupted growth in a D flask.

Comparative experiments were made with fresh embryonic heart. The fragments survived longer, and showed great activity at the same time that the mass of the tissue slowly decreased.

In other experiments, fragments of a pure strain of fibroblasts or of embryonic heart were placed in D-5 flasks in a medium composed of plasma and Tyrode solution containing 0.5 per cent embryonic

TABLE I.

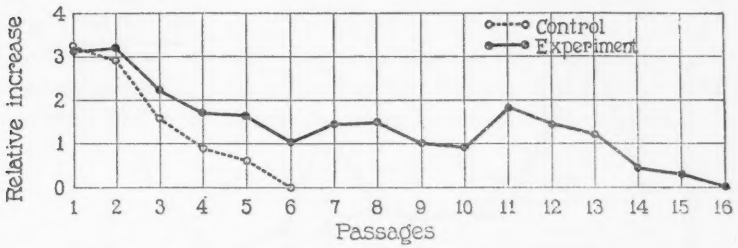
Action of 25 Per Cent Egg Albumin on Fibroblasts.

Passage No.	Experiment 1.		Experiment 2.			Experiment 3.			Experiment 4.			Experiment 5.	
	Culture No.	Relative increase.		Culture No.	Relative increase.		Culture No.	Relative increase.		Culture No.	Relative increase.		Culture No.	Relative increase.	
		Tyrode solution.	Albumin.		Tyrode solution.	Albumin.		Tyrode solution.	Albumin.		Tyrode solution.	Albumin.		Tyrode solution.	Albumin.
1	446	2.92	2.24	4.04	3.12	459	3.30	3.25	3.40	3.46	3.64	3.83			
2	456	2.86	*3.87	3.66	*4.14	470	3.40	*3.35	2.80	*3.46	1.90	*2.50			
3	469	2.37	2.86	2.33	2.77	480	1.60	2.40	1.19	1.50	0.88	1.60			
4	479	0.81	1.59	1.07	1.49	497	1.23	1.44	1.04	2.92	0.53	1.33			
5	496	0.63	1.70	0	1.56	512	0	1.38	0	1.90	0	1.76			
6	511	0	1.17		1.06	529		1.12		0.92		1.09			
7	528		0.84		0.82	550		2.00		1.87		1.78			
8	549		1.84		1.54	567		1.04		1.65		1.38			
9	566		1.38		1.13	589		1.06		0.74		0.91			
10	588		1.53		0.75	615		0.89		0.80		0.64			
11	614		0		0.65	635		3.00		1.83		2.00			
12	634				1.50	658		0.93		0.74		2.66			
13	657				0	676		1.50		0.80		1.20			
14						694		0.18		0.30		1.00			
15						711		0		0.30		0			
16						724				0					

* Culture divided; ½ discarded.

tissue juice. In the controls, 1 per cent Tyrode solution was poured into the flask after coagulation had taken place. Every 48 hours, the fluid was aspirated and replaced by the same amount of fresh Tyrode solution. In the experiments, the substance to be tested was substituted for Tyrode solution. The increase in area of the tissue, while in a condition of survival in the non-nutrient medium, was expressed by an S-shaped curve. The growth generally stopped after 7 or 8 days (Text-fig. 2).

2. Action of Egg Albumin on Pure Cultures of Fibroblasts.—Solutions of 25 and 35 per cent egg white in Tyrode solution were used. In the cultures containing 25 per cent egg white, the migration of fibroblasts was more active than in the controls containing only Tyrode solution



TEXT-FIG. 3. Action of a 25 per cent solution of egg albumin on pure cultures of fibroblasts.

TABLE II.

Action of 35 Per Cent Egg Albumin on Fibroblasts.

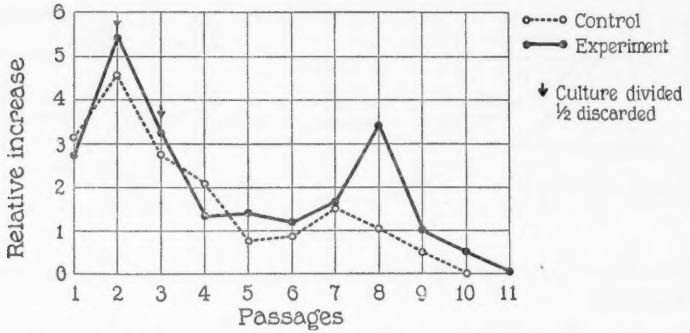
Passage No.	Culture No.	Experiment 1. Relative increase.		Experiment 2. Relative increase.		Experiment 3. Relative increase.	
		Tyrode solution.	Albumin.	Tyrode solution.	Albumin.	Tyrode solution.	Albumin.
1	520	3.00	2.87	3.12	2.89	3.31	2.88
2	539	4.74	*4.86	4.00	*5.62	5.00	*5.72
3	551	2.84	*3.47	2.59	*2.74	2.89	*3.84
4	568	2.10	1.32	2.10	1.57	2.00	1.21
5	590	0	1.27	0.78	1.30	0.70	1.69
6	616		2.00		0.78	0.85	0.84
7	636		1.89		1.69	1.50	1.44
8	659		3.27		3.75	1.08	3.25
9	677					0.50	1.00
10	695					0	0.50

* Culture divided; ½ discarded.

(Table I). The mass of the tissues appeared to increase, and once the tissue fragments had to be divided (Text-fig. 3). But there was no further increase in the bulk of the tissues. The migration remained very active, and the decrease in the size of the tissues was slower than in Tyrode solution. While the control died after five or six passages,

the life of the tissues cultivated in egg albumin lasted for fifteen passages, that is, for about 30 days (Text-fig. 3).

Similar experiments were repeated with egg white of a concentration of 35 per cent (Table II and Text-fig. 4). The duration of life was



TEXT-FIG. 4. Action of a 35 per cent solution of egg albumin on pure cultures of fibroblasts.

TABLE III.

Action of Egg Yolk on Fibroblasts.

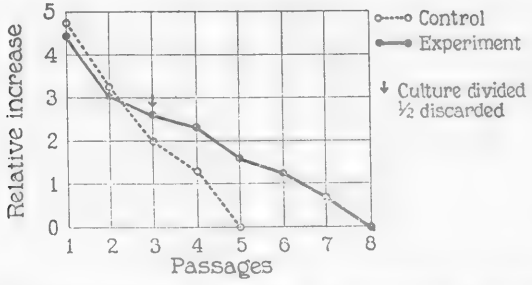
Passage No.	Culture No.	Experiment 1. Relative increase.		Experiment 2. Relative increase.		Experiment 3. Relative increase.	
		0.25 per cent egg yolk.	2.5 per cent egg yolk.	0.25 per cent egg yolk.	2.5 per cent egg yolk.	0.25 per cent egg yolk.	2.5 per cent egg yolk.
1	434	5.03	4.06	4.40	4.95	4.89	4.35
2	445	3.38	2.89	3.00	2.74	3.42	3.54
3	455	1.77	2.10	2.10	3.05	2.10	2.64
4	468	1.26	*2.23	0	*2.30	1.50	*2.38
5	481	0	1.56		1.42	0	†1.84
6	499		1.63		0.81		
7	510		0.77		0.60		
8	527		0		0		

*Culture divided; ½ discarded.
†Culture infected; discarded.

slightly shorter than in the 25 per cent solution. In solutions of higher concentrations than 35 per cent, the growth was very much less.

In order to ascertain whether the favorable effect of egg white is due to the albumin or to some other substance, the effect on fibroblasts

of pure albumin prepared by the methods of Sörensen and Hopkins was ascertained. A solution containing 5 per cent egg albumin prepared by Dr. Fischer according to the Sörensen method was tested on fibroblasts. The medium was made of 1 volume of plasma and 2 volumes of egg albumin. The rate of growth of the fibroblasts was slower than in the control composed of Tyrode solution. Similar experiments were repeated with a solution of egg albumin prepared according to the Hopkins method by Dr. Baker. The rate of growth was also found to be slightly decreased in the albumin solution.

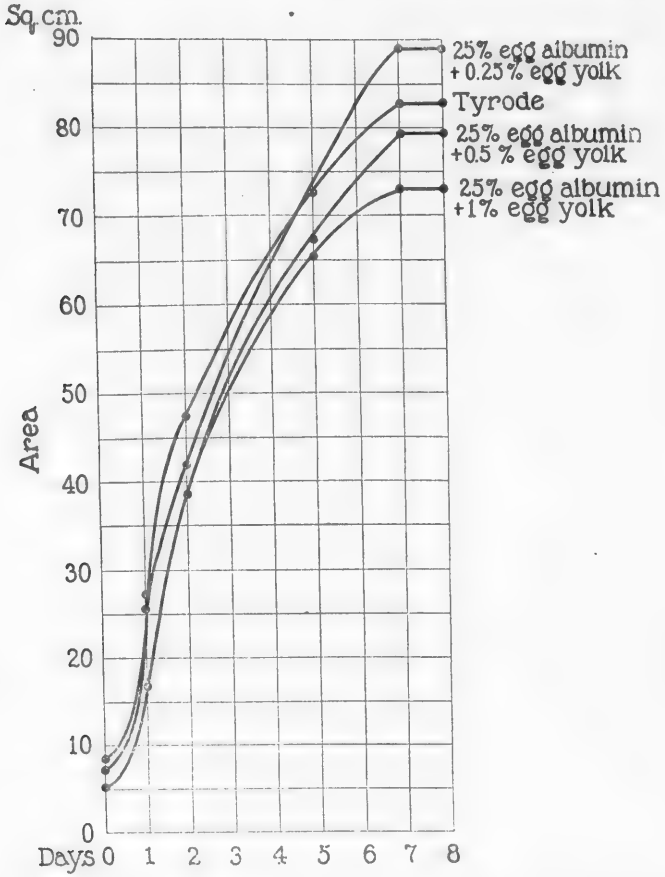


TEXT-FIG. 5. Action of a 2.5 and a 0.25 per cent solution of egg yolk on pure cultures of fibroblasts.

3. Action of Egg Yolk on Pure Cultures of Fibroblasts.—The effects on pure cultures of fibroblasts of two media containing 0.25 per cent and 2.5 per cent egg yolk were compared (Table III). There was a slight increase in the mass of the tissue and in the duration of life in the medium containing the larger amount of egg yolk. Although the tissues in the controls died after four passages, those in the experiments lived for six or seven passages (Table III and Text-fig. 5).

The effect of higher concentrations of egg yolk on fibroblasts was studied with a different technique. 1 cc. of suspensions of egg yolk in Tyrode solution in concentrations of 5 and 10 per cent respectively was injected into D-5 flasks containing fragments of embryonic heart imbedded in the solid medium. Every 48 hours, the fluid was replaced by a fresh solution. The rate of growth was no greater than in the preceding experiments, showing that fibroblasts are incapable of utilizing egg yolk for growth.

In a final series of experiments, the combined action of egg white and egg yolk was investigated. The fluid medium of D-5 flasks con-



TEXT-FIG. 6. Simultaneous action of egg yolk and egg white on pure cultures of fibroblasts in continuous growth in a D flask.

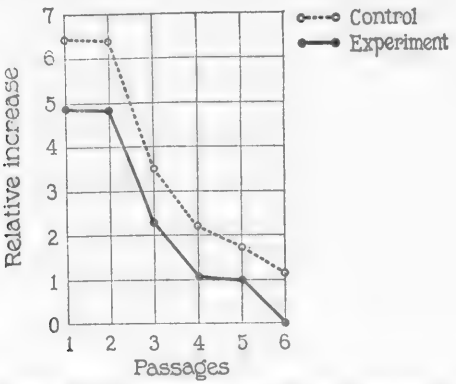
tained 25 per cent egg white and 1, 0.5, and 0.25 per cent, respectively, egg yolk. The rate of growth and the duration of life of the tissues were not increased (Text-fig. 6).

4. Action of Chicken Bouillon on Pure Cultures of Fibroblasts.— Bouillon was prepared from chicken tissues and added to the medium in concentrations varying from 10 to 20 per cent. In the cultures containing 20 per cent bouillon, the amount of growth was markedly

TABLE IV.

Action of 20 Per Cent Bouillon on Fibroblasts.

Passage No.	Culture No.	Experiment 1. Relative increase.		Experiment 2. Relative increase.		Experiment 3. Relative increase.		Experiment 4. Relative increase.	
		Tyrode solution.	Bouillon.	Tyrode solution.	Bouillon.	Tyrode solution.	Bouillon.	Tyrode solution.	Bouillon.
1	746	6.94	4.41	6.47	5.13	7.54	4.77	5.00	5.31
2	755	6.30	5.50	6.34	4.34	6.35	4.95	6.60	4.78
3	771	3.25	2.53	2.97	2.22	3.24	2.54	2.75	1.89
4	779	2.85	0.74	1.90	1.80	1.96	1.54	1.94	0.23
5	796	1.63	0	1.67	0.89	1.65	1.20	1.89	0
6				1.16	0	1.21	0		



TEXT-FIG. 7. Action of 20 per cent bouillon on pure cultures of fibroblasts.

decreased and also the duration of life (Table IV and Text-fig. 7). Concentrations below 10 per cent, such as used by Lewis and Lewis in some of their experiments with nutrient agar,[4] had no retarding effect. The growth of the cultures in bouillon was about equal to that of the

[4] Lewis, M. R., and Lewis, W. H., *Bull. Johns Hopkins Hosp.*, 1911, xxii, 126.

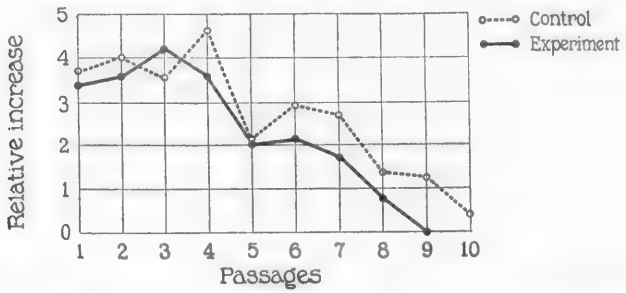
cultures in Tyrode solution. The duration of life was not affected (Table V and Text-fig. 8).

In other experiments, a bouillon was made of embryonic tissue juice, heated in a sealed tube at 100°C. for 1 hour. It had a slightly retarding action on the multiplication of fibroblasts.

TABLE V.

Action of 10 Per Cent Bouillon on Fibroblasts.

Passage No.	Culture No.	Experiment 1. Relative increase.		Experiment 2. Relative increase.		Experiment 3. Relative increase.		Experiment 4. Relative increase.	
		Tyrode solution.	Bouillon.	Tyrode solution.	Bouillon.	Tyrode solution.	Bouillon.	Tyrode solution.	Bouillon.
1	672	3.40	4.00	3.89	3.10	3.60	3.09	3.91	3.38
2	685	3.23	3.95	5.00	3.00	4.00	3.50	3.95	3.60
3	701	2.94	4.00	3.50	3.93	3.66	4.46	4.00	4.45
4	718	4.75	3.00	4.00	3.50	4.64	2.66	4.64	5.10
5	731	2.58	2.34	2.16	3.00	1.71	1.56	1.60	1.28
6	745	3.15	2.00	2.61	2.13	2.85	2.00	3.00	2.36
7	754	2.57	0	2.87	0	2.25	1.53	2.86	2.00
8	770	1.33		1.70		2.00	0	1.10	0.75
9								1.24	0
10								0.39	



TEXT-FIG. 8. Action of 10 per cent bouillon on pure cultures of fibroblasts.

DISCUSSION AND CONCLUSIONS.

When multiplying actively in serum, fibroblasts are in a condition of survival, not of cultivation, for they do not synthetize protoplasm from substances contained in the medium. They are able to utilize

only the nitrogenous material stored in the tissue itself. The distinction between survival of tissue and active growth is an important one. Innumerable errors in the interpretation of the experimental results have been made during the past years on account of this lack. An active proliferation of fibroblasts taking place during several days in a medium containing a given substance does not mean that this substance is used by the cells, but indicates only that residual cell proliferation is unopposed by a toxic substance. It is not possible to ascertain whether a medium is nutrient or not in the sense that it supports growth unless the mass of the tissues is shown to increase. When the proliferating fragment can be halved again and again, and each half reaches the size of the original whole in 48 hours, it is evident that new protoplasm must have been synthetized from the medium. The experiments described above show that egg white, egg yolk, pure egg albumin, egg white mixed with egg yolk, and bouillon are not utilized by fibroblasts for multiplication. This fact renders more plausible the hypothesis advanced previously that for proliferation the fibroblasts require some nitrogenous substances elaborated by other cells, which are present in embryonic tissue juices.

It may be concluded that, under the conditions of the experiments:

1. The duration of life of pure cultures of fibroblasts in a nitrogen-free medium is about 8 days.

2. Egg white and egg yolk, isolated or together, slightly increase the activity of the fibroblasts, but this effect is temporary and the tissues ultimately die. Pure egg albumin and bouillon are not utilizable by fibroblasts for multiplication.

ACTION ON FIBROBLASTS OF EXTRACTS OF HOMOLOGOUS AND HETEROLOGOUS TISSUES.

By ALEXIS CARREL, M.D., and ALBERT H. EBELING, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, June 5, 1923.)

Fibroblasts do not multiply in blood serum[1] after the food material stored in the original tissue is exhausted.[2] Neither can they obtain the nitrogen necessary for their proliferation from chicken bouillon, egg white, egg yolk, nor pure egg abumin.[3] But they live and multiply indefinitely when the medium contains a small amount of embryonic tissue juice.[4] It is, therefore, probable that they require for the building up of protoplasm certain nitrogenous compounds which are synthetized within the organism by other cells. It is known that leucocytes contain such growth-promoting substances.[5] Extracts of muscle and gland tissues of adult animals also possess the power of stimulating the rate of growth of homologous fibroblasts *in vitro*.[6] The juice of heterologous tissues, such as guinea pig tissue, may determine the proliferation of chicken fibroblasts, although the life of these cells in such a medium is not permanent.[7] It seemed that heterologous as well as adult tissues contain certain

[1] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 317; 1923, xxxvii, 759.

[2] Lewis, M. R., and Lewis, W. H., *Anat. Rec.*, 1911, v, 277. Ingebrigtsen, R., *J. Exp. Med.*, 1912, xvi, 421. Burrows, M. T., *Anat. Rec.*, 1916–17, xi, 335. Burrows, M. T., and Neymann, C. A., *J. Exp. Med.*, 1917, xxv, 93. Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 317.

[3] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1923, xxxviii, 487.

[4] Carrel, A., *J. Exp. Med.*, 1912, xv, 516. Ebeling, A. H., *J. Exp. Med.*, 1922, xxxv, 755.

[5] Carrel, A., *J. Exp. Med.*, 1922, xxxvi, 385. Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1922, xxxvi, 645.

[6] Carrel, A., *J. Exp. Med.*, 1913, xvii, 14.

[7] Carrel, A., unpublished experiments.

substances which permit a pure culture of fibroblasts to increase in mass, at least for a time. The aim of the experiments described in this article was to determine as accurately as possible, with the techniques at our disposal, the action of homologous adult tissue extracts and of heterologous embryonic juices and adult tissue extracts on the rate of growth of chicken fibroblasts and on the duration of their life *in vitro*.

EXPERIMENTAL.

Young embryos of chickens, mice, guinea pigs, and rabbits were finely pulped and centrifuged. The supernatant juice was diluted with approximately its volume of Tyrode solution. Muscle and organs of adult chickens, mice, guinea pigs, and rabbits were sliced in a Latapie apparatus, and to the pulp was added its volume of Tyrode solution. After a few hours, the mixture was centrifuged and the supernatant fluid removed.

The juices and extracts were kept in a refrigerator and their H ion concentration was determined daily by the colorimetric method. The pH was about 7.8, and after a few days it became lower, especially in extracts of adult tissues. It was kept constant by the addition of small amounts of sodium hydrate. The juices and extracts were always used within a week after they had been prepared.

The action of the juices and extracts was tested on an 11 year old strain of fibroblasts. The stock cultures were divided into two equal parts. One of the fragments was placed in a medium composed of 1 volume of plasma and 2 volumes of Tyrode solution containing 0.02 of chick embryo juice, and used as a control, while the other fragment was placed in 1 volume of plasma and 2 volumes of tissue extract or tissue juice. The fragments were traced in a projectoscope after 1 and 48 hours and the relative increase was measured by the usual technique.[8] If, after 48 hours, the tissue fragments had doubled in size, they were divided into two equal parts, one half being discarded, and the remaining half cultivated in the same medium. When no marked increase had occurred, the fragments were merely washed and transferred to a fresh medium. The figures on relative increase of the fragments, their absolute dimensions at the beginning of each passage, and the number of passages during which they lived, made it possible to ascertain the action of the various juices and extracts on cell proliferation. The data for each experiment were tabulated, but to save space the tables have been omitted. For each group of experiments, a chart shows the variations in the relative increase of the tissues in function of the time, which is expressed by the number of passages.

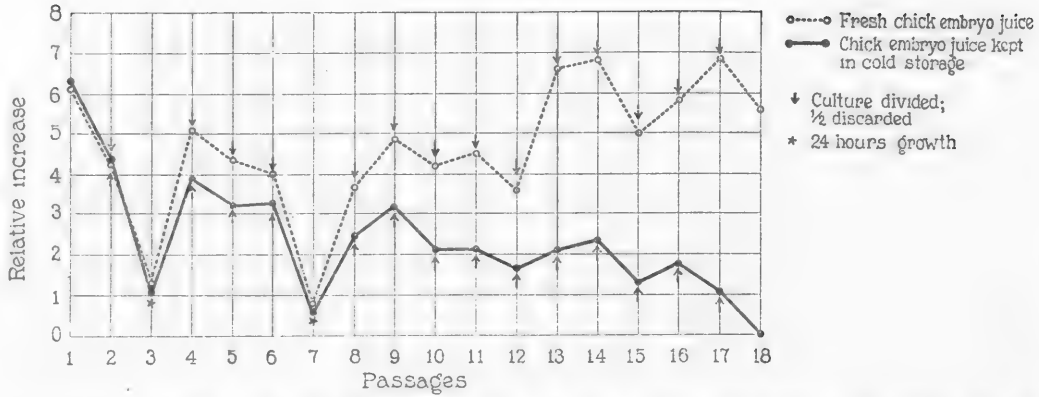
1. Action of Fresh Chick Embryo Juice and of the Same Juice Preserved in Cold Storage on the Duration of Life of Homologous

[8] Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 231.

Fibroblasts.—Before studying the action of the various homologous and heterologous juices on the growth of chicken fibroblasts, it was necessary to ascertain in more precise manner than has been done heretofore the influence of the spontaneous modifications undergone by the juices in function of time. It is well known that the increase in the H ion concentration prevents the stimulating action of the juice on cell proliferation,[9] and that this increase occurs spontaneously. But as it is also possible that some other deterioration takes place, even when the pH does not vary, the action of fresh juices was compared with that of juices kept in cold storage at a constant pH. In the following experiments, fresh juice and juice kept in cold storage were compared. When the juice had been kept in the refrigerator for more than 1 week, its action became weaker than that of the fresh extract. The tissues which had been cultivated in the juice kept for 34 days in the refrigerator died after seventeen passages, while those cultivated in fresh extract continued doubling in size every 48 hours (Text-fig. 1). It was not possible to determine whether the cause of death was intoxication or starvation. This experiment showed that, when the H ion concentration was kept constant by the addition of sodium hydrate, some changes occurred in the embryonic tissue juice which rendered it unsuitable for maintaining fibroblasts in a condition of indefinite growth. But this effect could hardly have been detected if the tissues had not been in contact for several passages with the juice. Tissue juices and extracts should be used, therefore, when freshly prepared.

2. Action of Extracts of Adult Homologous Tissues on the Growth and Duration of Life of Fibroblasts.—The extracts made from muscle brought about an increase in the mass of the tissues during the first passage. In one group of three experiments, the tissues could be divided into two parts at the third passage (Text-fig. 2), and apparently the muscle tissue extract had an action similar to that of embryonic tissue juice. However, it was observed that the cells contained more fat granules than do normal cultures. After either four or five passages, the tissues died. The controls in Tyrode

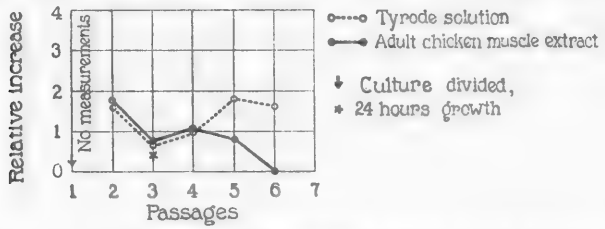
[9] Fischer, A., *J. Exp. Med.*, 1921, xxxiv, 447.



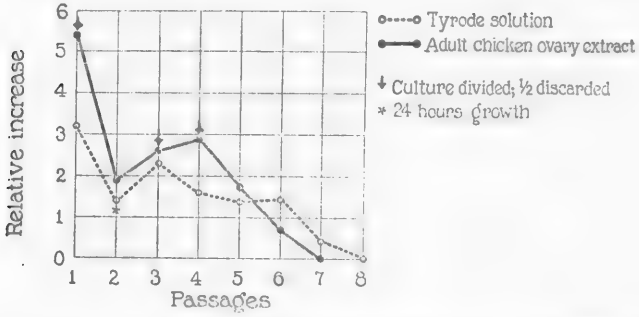
TEXT-FIG. 1. Action of fresh chick embryo juice and of chick embryo juice kept in cold storage on the rate of growth of homologous fibroblasts.

solution did not increase in size to such an extent that they could be divided. They were still living after six passages (Text-fig. 2).

In one group of three experiments, extracts of ovary also had a stimulating action. The mass of the tissues increased, but not permanently. The cultures were large enough to be divided after the first, third, and fourth passages. Death occurred at the seventh



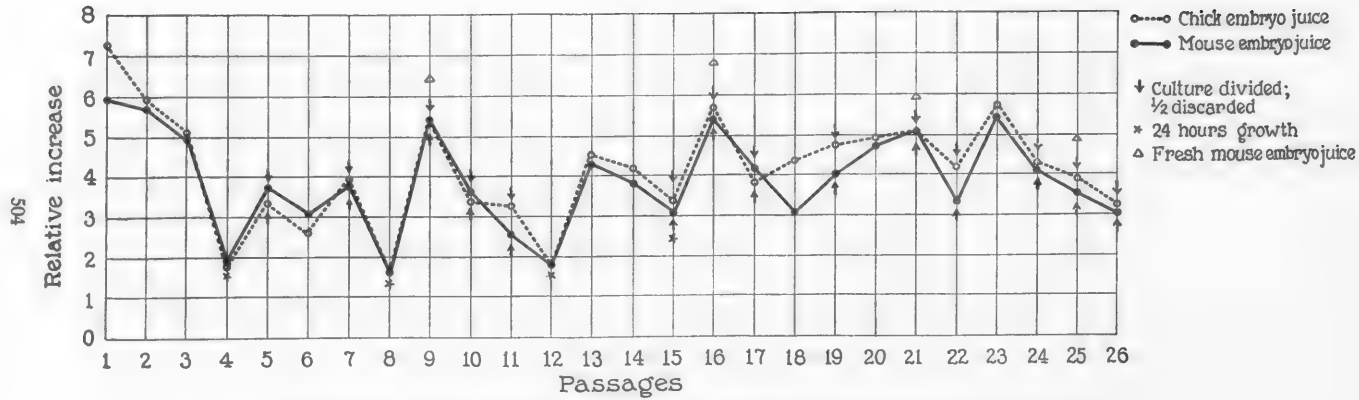
TEXT-FIG. 2. Action of adult chicken muscle extract on the duration of life of homologous fibroblasts.



TEXT-FIG. 3. Action of adult chicken ovary extract on the duration of life of homologous fibroblasts.

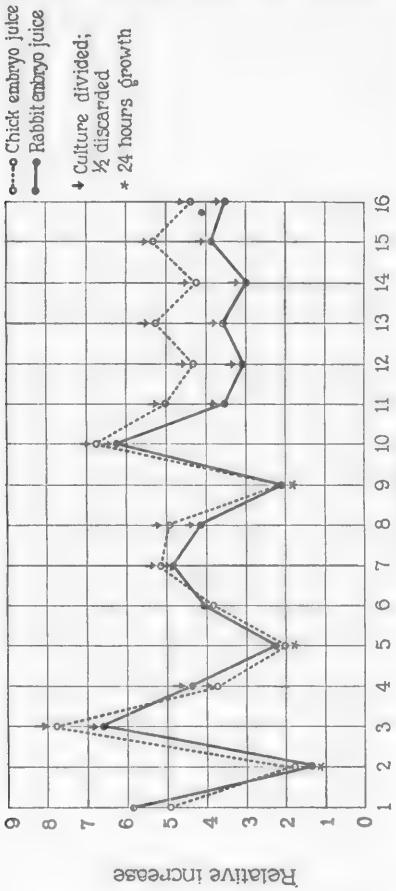
passage. The controls did not increase in mass and could not be divided, but they survived seven passages (Text-fig. 3). Thus, it appeared that the extracts of adult tissues were not capable of maintaining indefinitely the life of the fibroblasts *in vitro*, as fresh embryonic juice does.

3. Action of Heterologous Embryonic Tissue on the Growth and Duration of Life of Fibroblasts.—In one group of three experiments,



TEXT-FIG. 4. Action of mouse embryo juice on the rate of growth of chicken fibroblasts.

the action of the juice of mouse embryos was tested on chicken fibroblasts, the controls being cultivated in chick embryo juice. The supply of mouse embryo juice was renewed after 12, 26, 36,

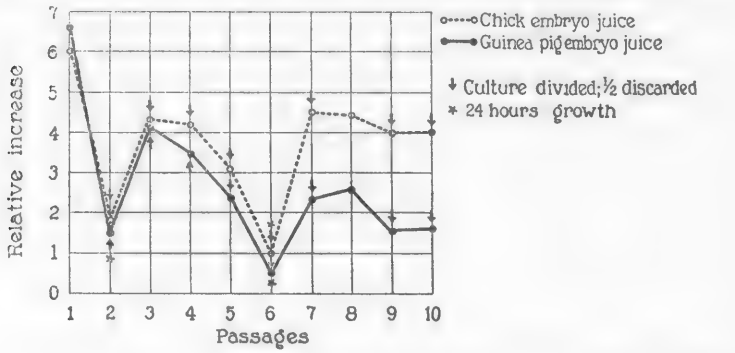

Chick embryo juice
Rabbit embryo juice
Culture divided; ½ discarded
* 24 hours growth
Relative increase
Passages
0 1 2 3 4 5 6 7 8 9
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

TEXT-FIG. 5. Action of rabbit embryo juice on the rate of growth of chicken fibroblasts

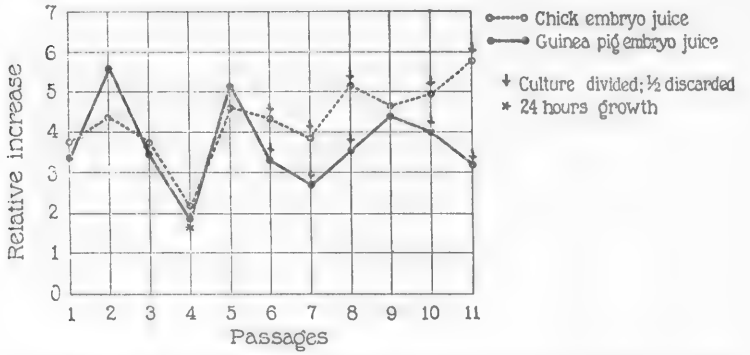
and 44 days. Text-fig. 4 shows that the connective tissue fragments doubled in mass in 48 hours in mouse embryo juice, as well as in chick embryo juice. After twenty-six passages, the rates of growth were still practically the same in both substances and the

experiment was discontinued. No evidence had been obtained that the tissues would die sooner in mouse embryo juice than in chick embryo juice.

In one group of three experiments, rabbit embryo juice acted on chicken fibroblasts in the same manner as chick embryo juice. The



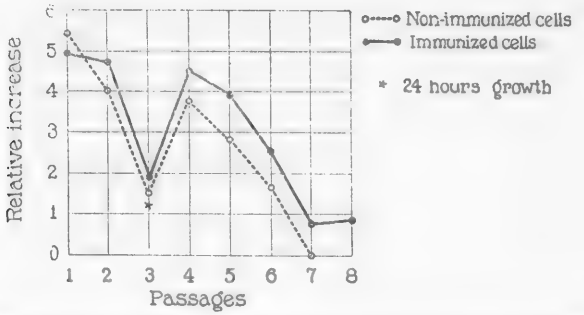
TEXT-FIG. 6. Action of guinea pig embryo juice on the rate of growth of chicken fibroblasts.



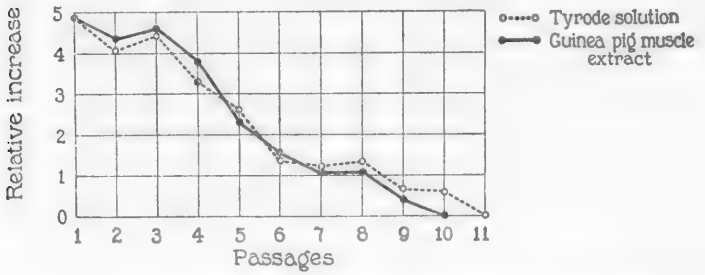
TEXT-FIG. 7. Action of guinea pig embryo juice on the rate of growth of chicken fibroblasts.

tissues increased markedly in size and no difference could be observed between fibroblasts nourished on chick embryo juice and rabbit embryo juice. Every 48 hours, the tissues doubled in size. This experiment was continued for 29 days. The curve shows that the

rates of growth of the fibroblasts in both juices were in general similar (Text-fig. 5). The differences observed after the 19th day were due probably to the fact that the supply of rabbit embryo juice had not been renewed. A similar slackening in the growth was observed when homologous tissue juice was used under the same conditions.



TEXT-FIG. 8. Action of rabbit serum on the rate of growth of chicken fibroblasts cultivated previously in chicken and rabbit embryo juices.

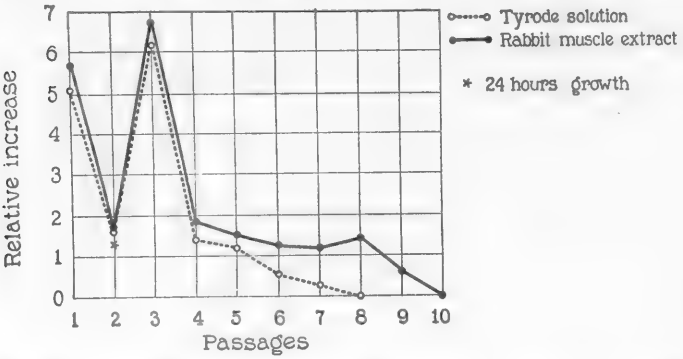


TEXT-FIG. 9. Action of adult guinea pig muscle extract on the duration of life of chicken fibroblasts.

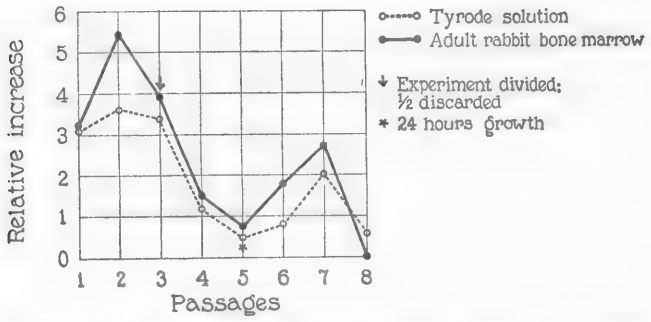
In two groups of three and four experiments respectively, the action of guinea pig embryo juice was studied in the same manner. In a first series of experiments, the tissues doubled in mass every 2 days in both media. After seven passages, growth was more extensive in the chick embryo juice because the supply of guinea pig juice had not been renewed (Text-fig. 6). In a second series of ex-

periments, after 20 days the rate of growth of chicken tissue fed on guinea pig juice was the same as that of the control (Text-fig. 7).

It is evident that protoplasm can be synthetized by chicken fibroblasts nourished on guinea pig, mouse, and rabbit embryonic tissues.



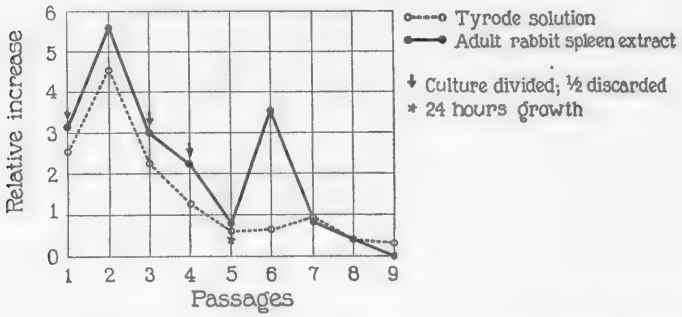
TEXT-FIG. 10. Action of adult rabbit muscle extract on the duration of life of chicken fibroblasts.



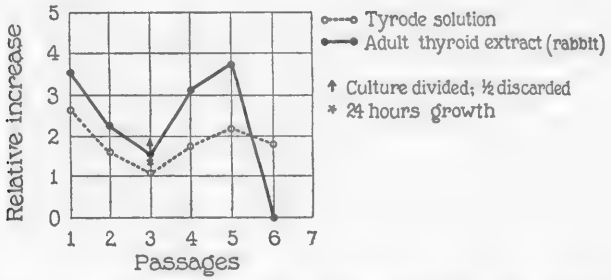
TEXT-FIG. 11. Action of adult rabbit bone marrow extract on the duration of life of chicken fibroblasts.

No differences traceable to variations in the media could be observed either in the rate of migration or the amount of tissue produced. Nevertheless, in one group of three experiments, an attempt was made to investigate whether chicken fibroblasts fed on rabbit juices were modified in their specificity. Strains of chicken fibroblasts

derived respectively from cultures maintained on chicken and rabbit embryo substances were cultivated in rabbit serum. The retarding action of rabbit serum was less marked on the fibroblasts grown in rabbit tissue juice than on the normal fibroblasts (Text-fig. 8). The fibroblasts previously nourished on rabbit tissue juice under-



TEXT-FIG. 12. Action of adult rabbit spleen extract on the duration of life of chicken fibroblasts.



TEXT-FIG. 13. Action of adult rabbit thyroid extract on the duration of life of chicken fibroblasts.

went but a slight change. This effect may have been due to the fact that the specificity of the chicken fibroblasts so nourished had decreased, or that they had become immunized against rabbit specific substances.

4. Action of Heterologous Adult Tissue Extract on the Growth and Duration of Life of Fibroblasts.—In one group of four experiments,

chicken fibroblasts cultivated in extract of adult guinea pig muscle did not increase in mass, and died sooner than the controls in Tyrode solution (Text-fig. 9).

In one group of three experiments, the extract of adult rabbit muscle had a similar action. The fibroblasts died 4 days sooner in muscle extract than in Tyrode solution (Text-fig. 10).

In one group of three experiments, rabbit bone marrow caused the fibroblasts to double in mass at the third passage. But both the experiment and control tissues died at the eighth passage (Text-fig. 11).

In one group of four experiments, rabbit spleen extract increased the mass of the tissues to the same extent, but the duration of life was no longer than in the controls (Text-fig. 12).

In one group of three experiments, extract of thyroid gland produced some increase in the mass of the tissues. Death occurred suddenly at the sixth passage (Text-fig. 13).

SUMMARY.

Extracts of homologous adult tissues determine an increase in the mass of pure cultures of chicken fibroblasts nourished thereon comparable to that resulting from embryonic tissue juice. But the effect of these extracts differs markedly from that of the latter, since cell multiplication does not continue indefinitely. After a few passages, the fibroblasts cultivated in adult tissue extracts grew more slowly than in Tyrode solution. The cytoplasm became dark and filled with fat granules, and death followed. It is possible that the tissues of adult animals contain, as does the serum, substances which are toxic for the homologous cells, and which progressively overcome the effect of the growth-activating substances. The effect of heterologous adult tissue extracts did not differ markedly from that of homologous tissues. The chicken connective tissue increased slightly in mass, but died sooner than the controls in Tyrode solution.

By contrast, tissue juices derived from the embryos of mice, guinea pigs, and rabbits acted on chicken fibroblasts in the same manner as chick embryo juices. The increase in mass of the cultures was regular and rapid. They doubled in size every 48 or 72 hours, and the

rate of growth did not decrease after 30 days. It appears that embryonic tissue juices are not necessarily toxic for heterologous fibroblasts, and that they can be used in the building up of protoplasm in the tissues of a different species. In experiments made long ago,[6] the action of tissue juice was described as being specific. The premature death of the fibroblasts cultivated in heterologous juices at that time would now appear to have been due to spontaneous changes in the pH and the deterioration that even normal chick embryo juice at a pH of 7.8 undergoes spontaneously. In the recent experiments, when freshly prepared homologous and heterologous juices were used, their action on chicken fibroblasts in pure culture was identical. However, the fibroblasts produced in cultures nourished by rabbit juice grew better when transferred to rabbit serum than did ordinary chicken fibroblasts. It has not been determined as yet whether this effect is due to an immunization of the fibroblasts against rabbit humors, or to some decrease in the specificity eventuating in cells intermediate between rabbit and chicken fibroblasts.

It may be concluded that, under the conditions of the experiments:

1. Pure cultures of chicken fibroblasts increase in mass under the influence of extracts of adult homologous tissues. But they ultimately die while the fibroblasts cultivated in embryonic tissue juices live indefinitely.

2. The increase in mass of chicken fibroblasts cultivated in the juices of mouse, guinea pig, rabbit, and chick embryos is about identical.

3. Chicken fibroblasts produced in cultures nourished by rabbit embryonic tissue juice are less sensitive to the inhibiting action of rabbit serum than ordinary chicken fibroblasts.

4. Cultures of chicken fibroblasts in extracts of adult tissues of mice, guinea pigs, and rabbits increase slightly in mass, but the increase is temporary and death occurs after a few passages.

ACTION OF SERUM ON LYMPHOCYTES IN VITRO.

By ALEXIS CARREL, M.D., and ALBERT H. EBELING, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

PLATES 32 AND 33.

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Fibroblasts do not obtain from serum,[1] egg white, pure egg albumin, bouillon,[2] or amino acids,[3] the nitrogen necessary for their nutrition. When cultivated with these substances, they fail to multiply even in the presence of egg yolk which contains the necessary accessory factors.[3] It appears that they require for their proliferation certain special substances synthetized by embryonic tissue.[1] There is evidence for the view that, *in vivo*, they obtain these elements from the white blood corpuscles which possess the property of manufacturing and secreting them.[4] Should this conjecture be true, leucocytes must be capable of transforming some of the serum constituents into the more complex compounds necessary for the nutrition of the fibroblasts and epithelial cells. Therefore, an attempt was made to ascertain whether lymphocytes and large mononuclear cells multiply in a medium containing serum as the sole source of nitrogen, and set free substances determining the proliferation of a pure strain of fibroblasts.

EXPERIMENTAL.

1. Action of Serum on the Multiplication of Lymphocytes in Vitro.—Fragments of embryo spleen or of a coagulum containing white blood corpuscles were imbedded in a medium consisting of 1 cc. of chicken plasma, 1.5 cc. of Tyrode solution containing 10 per cent embryonic

[1] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 317; 1923, xxxvii, 759.

[2] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1923, xxxviii, 487.

[3] Carrel, A., and Ebeling, A. H., unpublished experiments, 1923.

[4] Carrel, A., *J. Exp. Med.*, 1922, xxxvi, 385. Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1922, xxxvi, 365, 645.

tissue juice, and 1.5 cc. of Tyrode solution, according to a technique previously described.[5] After 2 days incubation, 1 cc. of serum diluted with its volume of Tyrode solution was introduced into the flask. After 3 or 4 days, the fluid was aspirated and replaced by 1 cc. of pure Tyrode solution for 24 hours when diluted serum was again placed in the flask.

After a few hours, a great many ameboid cells wandered from the original fragment and invaded the culture medium. The polymorphonuclear cells disappeared after a short time, and lymphocytes and large mononuclear cells migrated through the coagulum as individual units without any tendency to form a tissue. As the experiments were performed early in the development of the special culture method utilized, several contaminations or other accidents occurred, and only a few cultures were successfully observed for more than a month.

Two cultures were prepared in D flasks, 5 cm. in diameter, which contained one and three fragments of spleen about 1 sq. mm. in area. The migration and multiplication of the cells proceeded slowly. The rate of multiplication increased temporarily each time fresh serum was added to the medium. The wandering cells invaded the medium at first on one plane in an orderly fashion, each cell being separated from the neighboring cells by practically regular intervals. Later, they grew on three or four planes (Fig. 1), but without any contact or tendency to tissue formation. They accumulated around a few foreign bodies which were in the medium, as they generally do *in vivo*. After 30 days, practically the entire medium was invaded by the wandering cells. They covered a circle about 45 mm. in diameter, that is, an area of about 2,000 sq. mm. As the surface area of the original fragment of spleen was about 1 sq. mm., it was evident that the lymphocytes must have multiplied. In other cultures, small islands of lymphocytes appeared at a distance from the original fragment of spleen. The growth of these islands could be readily observed, showing that the spreading of the cells is due to the multiplication, and not merely to the migration, of cells contained in the original fragment of spleen. There is no doubt that the number of lymphocytes increased immensely during their cultivation in serum.

[5] Carrel, A., *J. Exp. Med.*, 1923, xxxviii, 407.

One of the cultures died of bacterial contamination after 36 days. As soon as the infection was detected, the fluid part of the medium was removed, the cultures were washed in Tyrode solution, fixed in formalin, and stained with eosin-azure. Many of the cells were dead and had become round when the culture was fixed. No polymorphonuclear elements were seen. All the cells appeared to be lymphocytes and large mononuclear cells (Fig. 2), with fibroblasts at the site of the original fragment.

In the second culture, the coagulum became detached from the bottom of the flask after the 34th day. The cells were in full activity. Some of the fragments of the coagulum were fixed and stained. The cells proved to be mononuclears and were large and active (Figs. 3 and 4).

Similar experiments were made with leucocytes obtained from blood. After chicken blood had been centrifuged for 10 minutes, a few drops of a dilute solution of embryonic tissue juice were placed on the corpuscles. Coagulation occurred, and fragments from the film containing the white blood corpuscles were cultivated in D-5 flasks. The fluid part of the medium was composed of Tyrode solution containing 30 per cent normal serum, and was renewed every 3 or 4 days. After 21 days, cells resembling lymphocytes had invaded practically the entire culture medium. They stopped at about 0.5 cm. from the edge of the flask, and died 10 days later.

2. Action of Lymphocytes and Large Mononuclear Cells on Fibroblasts Contained in a Medium Composed of Serum.—Previous experiments have shown that when a fragment of spleen is placed at a short distance from a culture of fibroblasts, the rate of multiplication of the fibroblasts is markedly increased,[6] owing to the secretion of growth-promoting substances by the leucocytes. It was found also that leucocytes do not live more than 10 days in Tyrode solution.[6] This means that in a non-nutrient medium, they exhausted their reserves in from 7 to 10 days. It follows that, if leucocytes cultivated in blood serum for more than 10 days are able to increase in rate of migration and multiplication of fibroblasts, it would indicate that the material used by them in the elaboration of the growth-promoting substances must come from the serum.

[6] Carrel, A., unpublished experiments, 1922–23.

In a D-5 flask, a fragment of spleen was placed 12 mm. from a fragment of a pure culture of fibroblasts. As the fluid part of the medium was composed of pure serum, the fibroblasts stopped proliferating after 5 days. Meanwhile, the leucocytes slowly invaded the medium and reached the neighborhood of the colony of fibroblasts. By the 9th day, the fibroblasts had become very large and their cytoplasm was dark and loaded with fat granules. Serum was now added to the medium and the lymphocytes proliferated very actively and some of them reached the fibroblasts. During the

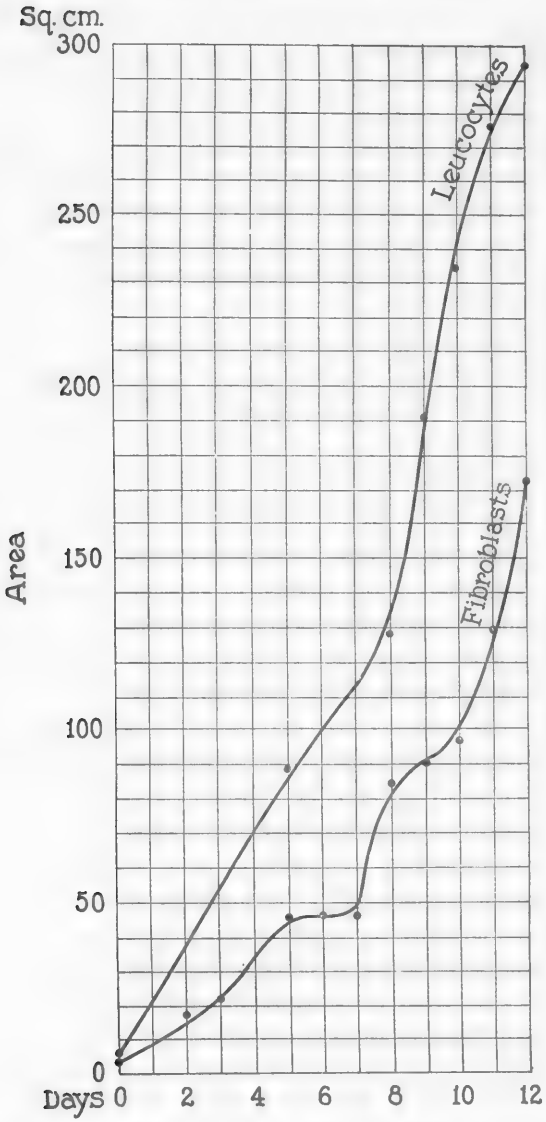
TABLE I.

Action of Lymphocytes on Fibroblasts in a Medium Composed of Serum.

Date.	Culture No.	Area of		Composition of fluid medium.
		Lymphocytes.	Fibroblasts.	
1923				
Feb. 19	2667–3	5.1	4.6	0
" 20				Serum 1 cc.
" 21			18.1	" 1 "
" 22			22.9	" 1 "
" 23				" 1 "
" 24		89.8	46.3	" 1 "
" 25			46.3	" 1 "
" 26			46.3	Tyrode 1 "
" 27		128.4	84.8	Serum 1 "
" 28		191.9	90.7	" 1 "
Mar. 1		234.5	97.5	" 1 "
" 2		276.4	128.5	Tyrode 1 "
" 3		294.1	172.9	Serum 1 "

following days, the appearance of the fibroblasts changed. They became slender and elongated at the same time that the cytoplasm decreased in volume, and they again began to proliferate actively, as indicated on the curve (Table I and Text-fig. 1). The number of leucocytes increased greatly and, on the 12th day, a great many began to die, owing probably to the accumulation of too large a number of cells in a comparatively small area. The fibroblasts were then quite refreshed and in full activity (Text-fig. 1).

A spontaneous rejuvenation of fibroblasts after they had been cultivated in serum for 8 days and had undergone degenerative



TEXT-FIG. 1. Action of lymphocytes on fibroblasts in the presence of serum.

changes has never been observed previously. It can be attributed to the nearby presence of lymphocytes acting upon the fibroblasts. The rate of proliferation of fibroblasts has been observed to increase several fold when they grow near colonies of lymphocytes. In the above experiment, an allied phenomenon took place. As it appeared after the lymphocytes had been cultivated in serum for more than 10 days, it may be assumed that the growth-promoting principle secreted by these latter cells must have been elaborated from substances contained in the serum, and not from their own reserves.

DISCUSSION.

These experiments have brought to light the following facts: lymphocytes are able to live and multiply in a medium containing only blood serum, which does not suffice for the purpose in the case of fibroblasts. The presence, under certain conditions, of lymphocytes and large mononuclear cells in the neighborhood of a culture of fibroblasts in serum markedly increases the activity and the duration of life of the fibroblasts.

Since lymphocytes and large mononuclear cells cultivated in serum proliferate and manufacture substances which enable fibroblasts to grow, they appear to be endowed with a function of primary importance in the nutrition of the tissues. It is known that fibroblasts and epithelial cells do not synthetize protoplasm when cultivated in serum, while they obtain certain substances from embryonic tissue juice which determine an immense proliferation. As leucocytes secrete substances possessing the same growth-activating properties as embryonic tissues, it was conjectured that fibroblasts and epithelial cells, elements which cannot survive permanently on the substances contained in serum, are maintained through the action of the white cells of the blood.[4] More than 30 years ago, Renaut was lead by his anatomical studies to assume that the lymphocytes bring to the fixed cells the food material which they need.[7] Recently, Jolly[8] concluded from his investigations of the influence of starvation on the lymphoid apparatus, that lymphocytes store important chemi-

[7] Renaut, J., Traité d'histologie pratique, Paris, 1893, i, pt. 2, 968.
[8] Jolly, J., *Compt. rend. Soc. biol.*, 1920, lxxxiii, 1209.

cal substances which are used by the organism more readily than are the materials from other tissues. Previous experiments[9] have shown that some of the substances secreted by leucocytes *in vivo* and *in vitro* have the same properties as embryonic tissue juice, and determine the multiplication of fibroblasts.

In the experiments described in this article, the source of the nitrogen used by the white cells in the manufacture of the essentials which promote the growth of the fibroblasts was investigated, and it appears that lymphocytes and large mononuclear cells find in blood serum all that is necessary for their proliferation and the elaboration of their secretions. They probably manufacture from certain compounds present in serum the food material which fibroblasts and epithelial cells require for their proliferation. Thus, they may be supposed to act as food transformers.

CONCLUSIONS.

It may be concluded that, under the conditions of the experiments:

1. Lymphocytes and large mononuclear cells can live and increase greatly in numbers in blood serum, while fibroblasts are not capable of doing so.

2. While living in serum, lymphocytes and large mononuclear cells manufacture and secrete substances which may be used as food material by the fibroblasts.

3. It is probable that lymphocytes and large mononuclear cells synthetize from the nitrogenous compounds contained in serum the substances which fibroblasts and epithelial cells require for their multiplication.

EXPLANATION OF PLATES.

PLATE 32.

FIG. 1. Living culture of lymphocytes and large mononuclears in a D-5 flask.

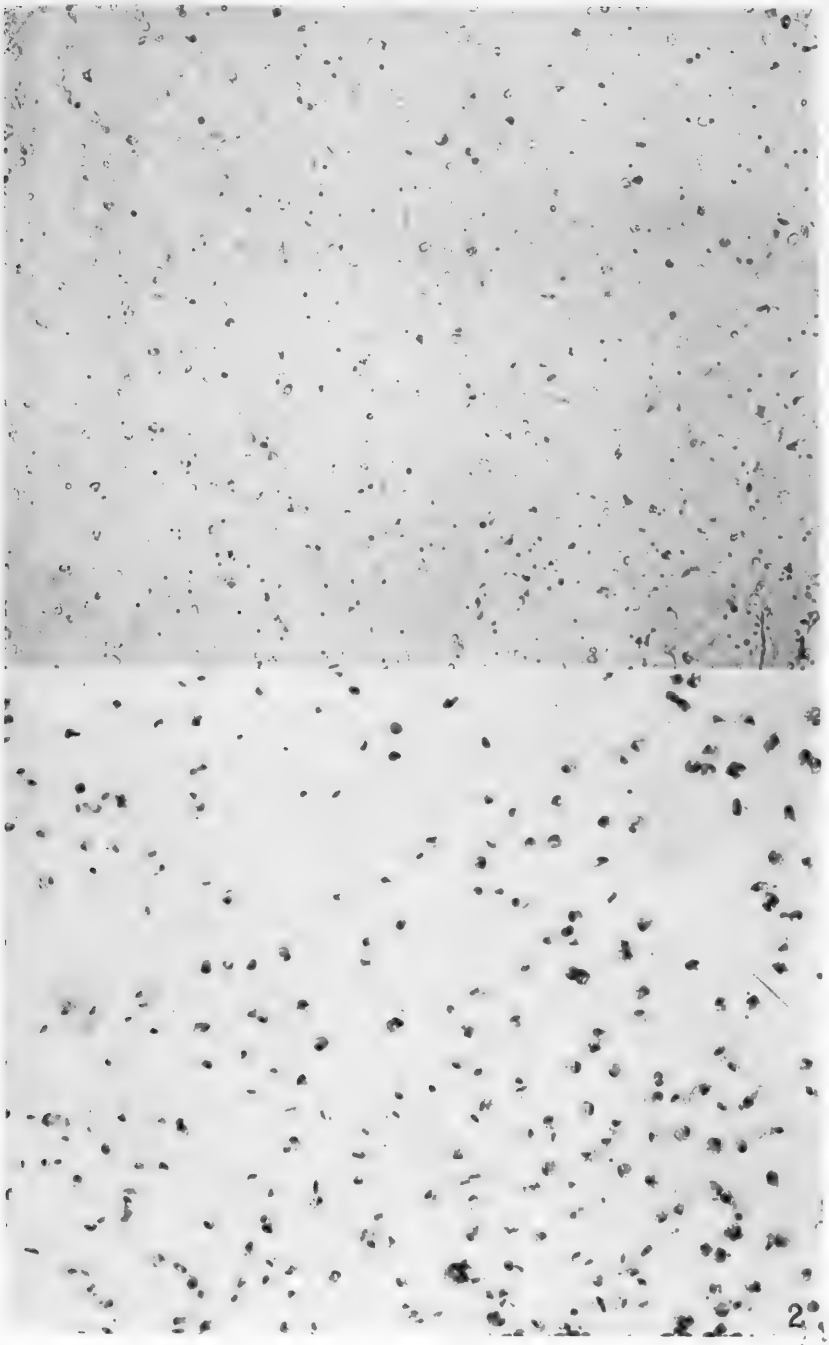
FIG. 2. 36 day old culture of lymphocytes in a D-5 flask.

PLATE 33.

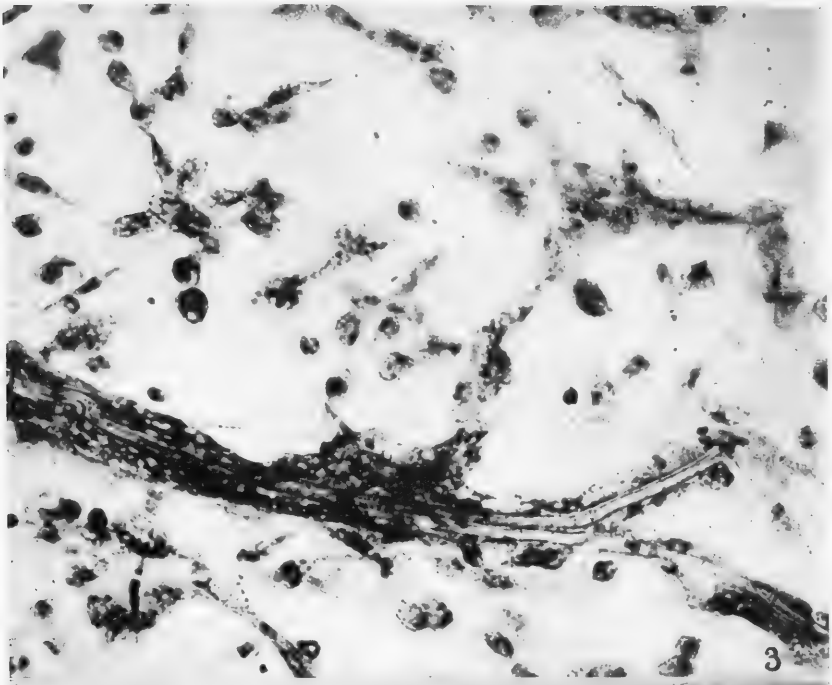
FIG. 3. 34 day old culture of lymphocytes showing encapsulation of a foreign body.

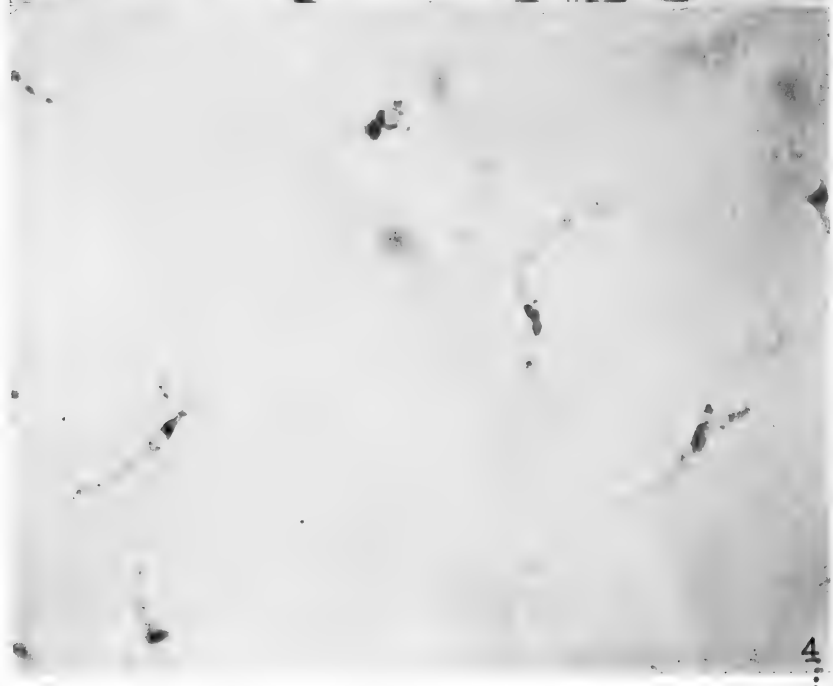
FIG. 4. Scattered mononuclears in the peripheral part of the same culture.

[9] Carrel, A., *J. Exp. Med.*, 1922, xxxvi, 385.



(Carrel and Ebeling: Action of serum on lymphocytes.)





(Carrel and Ebeling: Action of serum on lymphocytes.)

MEASUREMENT OF THE INHERENT GROWTH ENERGY OF TISSUES.

By ALEXIS CARREL, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

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It is well known that the growth energy of the organism decreases progressively with age.[1] The activity of each tissue probably declines at a different rate. Fibroblasts grow more rapidly *in vitro* from the heart of a young chick embryo than from that of an older one, and no cells migrate from a fragment of adult heart.[2] Cardiac muscle fibers lose the property of proliferating *in vitro* very early. Cultures of cutaneous epithelium can be obtained from an embryo, while no epithelial cells grow from the skin of a young chicken. Evidently, there is a definite relation between the growth energy of a given tissue and its age, which could be used for measuring the age of the organism. The rate of healing of an aseptic wound is a function of the age of the patient which is determined when the size of the wound and its index of cicatrization are known.[3] While the growth energy of the tissues slowly decreases in function of time, it may fluctuate under the influence of many other factors, as every physiological and pathological process involves a resumption or a decline of cell activity.

The variations of tissue activity *in vivo* are very probably related to those of the growth-inhibiting and promoting properties of the humors.[4] The decline of the rate of multiplication of fibroblasts cultivated in serum in function of the age of the animal from which the serum is obtained resembles the decrease of the index of cicatrization of a wound in function of the age of the patient.[5] The growth energy

[1] Minot, C. S., The problem of age, growth, and death, New York, 1908.

[2] Carrel, A., *J. Exp. Med.*, 1913, xviii, 287.

[3] du Noüy, P. L., *J. Exp. Med.*, 1916, xxiv, 461.

[4] Carrel, A., *J. Exp. Med.*, 1913, xvii, 14. Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 599; 1922, xxxvi, 365. Carrel, A., *J. Exp. Med.*, 1922, xxxvi, 385. Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1922, xxxvi, 645.

[5] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 599.

of the tissues decreases at the same time that the blood serum becomes more inhibiting to growth. Some years ago, it was found that fibroblasts in different conditions of activity, when cultivated in the same medium, soon grew at the same rate, and that if two halves of a culture were placed in media of different composition, they multiplied at different speeds after a short time.[2] This experiment demonstrated that the rate of cell proliferation *in vitro* depends on certain properties of the medium. We may, then, assume that the activity of a tissue *in vivo* at a given instant is probably a function of its activity at the preceding instant, and of the concentration of growth-activating and inhibiting substances in the interstitial lymph and the blood plasma.

The knowledge of the relations between the tissues and the humors would certainly be of great importance. We can already detect the presence of the growth-activating and growth-inhibiting substances in the blood and the lymph by determining the growth index of the fluids.[6] But a method for measuring inherent cell activity remains to be developed. This inherent growth energy is not improbably proportional to the residual growth energy, that is, to the energy spent by the tissues in a non-nutritive medium until death occurs. The purpose of the experiments described in this article was to develop a technique for measuring the residual energy of fibroblasts and the relations between inherent and residual energies.

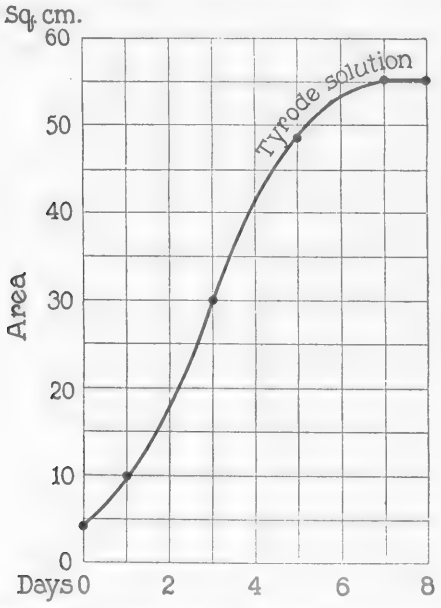
EXPERIMENTAL.

1. Measurement of the Residual Growth Energy of Fibroblasts.—A fragment of a pure strain of fibroblasts or of heart tissue was placed in a D-5 flask in 0.5 cc. of plasma and 1.5 cc. of Tyrode solution containing 5 per cent embryonic tissue juice.[7] After coagulation occurred, 1 cc. of Tyrode solution was poured on the surface of the coagulum. Every 48 hours, the fluid was aspirated and replaced by fresh Tyrode solution. Immediately after the preparation of the culture, and every 48 hours, the outline of the tissues was traced in a projectoscope, and the area measured with a planimeter. The extent of the migration of the fibroblasts and the duration of their life represented the residual

[6] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1922, xxxv, 17.
[7] Carrel, A., *J. Exp. Med.*, 1923, xxxviii, 407.

activity of the tissue, which finds expression ordinarily in an S-shaped curve.

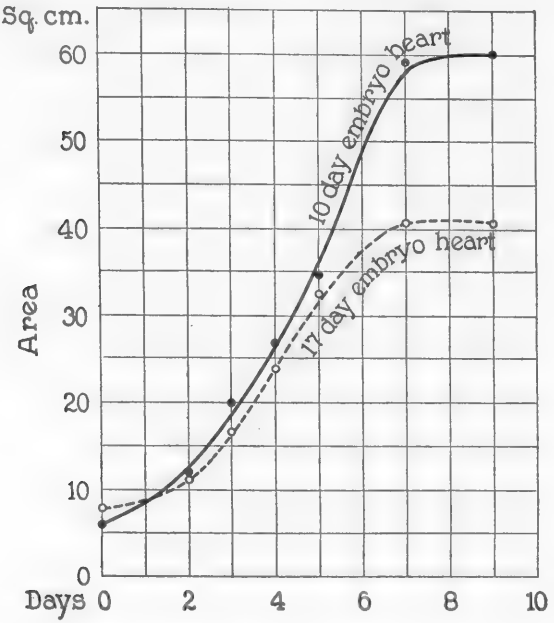
The fragments of fresh tissue usually died before the central part of the original portion disintegrated, that is, before the nutritive substances which they contained had been entirely used. The residual life of a fragment of heart of an 8 day embryo varied from 7 to 12 days. When the original fragment disintegrated early, the rate of multiplication and duration of the life of the fibroblasts were increased.



TEXT-FIG. 1. Residual growth energy of fibroblasts.

Fragments of a pure strain of fibroblasts used all the nutritive substances which they contained before they died. The residual life of a fragment of an 11 year old strain of fibroblasts, in normal condition, generally lasted 7 or 8 days (Text-fig. 1). Under the conditions of the experiments, the period of growth never extended beyond 9 or 10 days. During the first 2 days, there was very little difference in the rate of growth of fibroblasts cultivated in nutrient and non-nutrient

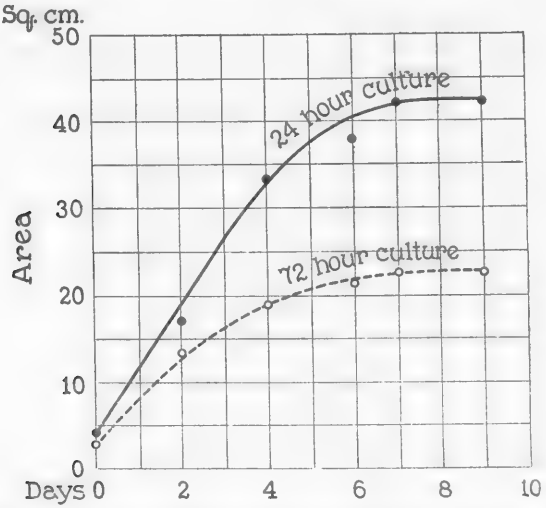
media. As the solid medium was composed of plasma, and the fluid medium of Tyrode solution renewed every 2 days, the tissues were bathed in a fluid which became progressively less concentrated in serum, that is, less inhibiting. The first inflection of the curve might be due to the dilution of the medium under the influence of the Tyrode solution. From the 2nd to the 4th or 5th day, the growth was very active. The effect of starvation became apparent after 4 days. The



TEXT-FIG. 2. Residual growth energy of fragments of heart of 10 and 17 day chick embryos.

growth slackened and the curve began the second inflection. As the solid medium was constantly in contact with Tyrode solution, the decrease in the rate of growth cannot be referred to an accumulation of catabolic substances, but to the lack of nutrient materials. It is evident that the activity of the cells in a medium composed chiefly of Tyrode solution must depend in large measure upon the preceding condition of activity.

2. Residual Growth Energy of Tissues from Animals of Different Ages.—In several experiments, two fragments of the ventricle of the hearts of 10 and 17 day chick embryos were placed in a D-5 flask, according to a technique previously described.[7] The curve expressing the residual activity, as determined by the experiment and recorded in Text-fig. 2, shows that the duration of life of both tissues was about the same, but that the growth of the older tissue was 30 per cent less



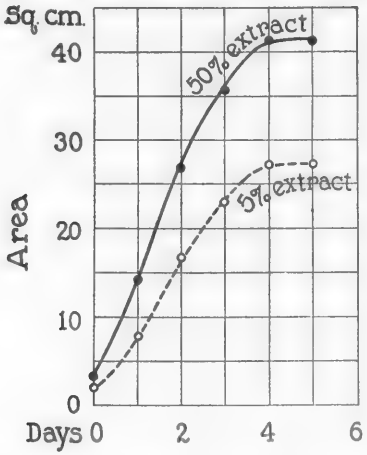
TEXT-FIG. 3. Residual growth energy of fibroblasts kept previously in a hanging drop for 24 and 72 hours respectively.

extensive than that of the younger. There was certainly a marked difference in the inherent activities of the two tissues referable to the difference in age of the embryos from which they were derived.

3. Residual Growth Energy of a Pure Strain of Fibroblasts after 24 and 72 Hours Cultivation in a Hanging Drop.—When fibroblasts are cultivated in a hanging drop of plasma and embryonic tissue juice, the S-shaped growth curve shows that great variations occur in the inherent activity of the tissues during 72 hours. The period of optimal growth lasts from about the 12th to the 48th hour. Later, the curve flattens

and becomes almost parallel to the time axis after 72 hours.[8] This second inflection is due to the spontaneous disappearance of the thermolabile growth-promoting substances of the medium and to the accumulation of catabolic products. It is probable that fragments of a pure strain of fibroblasts brought to different states of activity by cultivation for 24 and 72 hours in a hanging drop would show corresponding differences of residual activity.

Several fragments of an 11 year old strain of fibroblasts were cultivated on hollow slides in hanging drops composed of 1 volume of plasma and 1 volume of embryonic tissue juice. After 24 and 72 hours,



TEXT-FIG. 4. Residual growth energy of fibroblasts kept previously for 48 hours in a hanging drop containing 5 and 50 per cent embryonic tissue juice respectively.

respectively, fibroblasts were taken from the hanging drop cultures, imbedded in the medium in a D-5 flask, and their residual growth observed. The duration of life was about the same, but the extent of migration of the fibroblasts cultivated for 72 hours was about 45 per cent less than that of those cultivated for 24 hours (Text-fig. 3).

4. Residual Growth Energy of a Pure Strain of Fibroblasts Cultivated in Media Containing 5 and 50 Per Cent Embryonic Tissue Extracts.—Two halves of a fragment from an 11 year old strain of fibroblasts were placed

[8] Ebeling, A. H., unpublished experiments, 1919.

in media containing respectively 5 and 50 per cent embryonic tissue juice, in order to modify the inherent activity. After 48 hours, both fragments were placed in a flask in the ordinary medium and the residual activity was measured. The duration of life of both fragments was about the same, but the area covered by the cells of the fragments previously cultivated in 50 per cent embryonic tissue juice was much larger. In the experiment recorded in Text-fig. 4, the activity of the cells, as expressed by the extent of their migration, was 25 per cent greater for the tissues previously cultivated in 50 per cent embryonic tissue juice than for those cultivated in 5 per cent.

CONCLUSIONS.

1. The residual growth energy of fibroblasts is expressed by the extent of their migration and multiplication in a non-nutrient medium.

2. The residual energy of fibroblasts is related to their inherent energy and the variations of the inherent energy can be ascertained by the measurement of the residual energy.

BLOOD CELL FORMATION AND DISTRIBUTION IN RELATION TO THE MECHANISM OF THYROID-ACCELERATED METAMORPHOSIS IN THE LARVAL FROG.

By H. E. JORDAN, Ph.D., and C. C. SPEIDEL, Ph.D.

(*From the Department of Histology and Embryology, University of Virginia Medical School, Charlottesville.*)

Plates 34 to 36.

(Received for publication, June 5, 1923.)

A study of blood formation in the adult frog has shown that the spleen is the chief hemopoietic organ (Jordan and Speidel, 1923). Red bone marrow may appear for a brief period in the spring, following the hibernation period, and become a locus of hemopoietic activity as pointed out by Jordan (1919) for the leopard frog, but this condition does not persist for more than a few weeks. For the remainder of the year the spleen is practically the sole organ of blood formation. In the larval frog, however, the kidney plays an important part in this process. The intertubular regions of the tadpole kidney are packed with many erythrocytes and leucocytes, together with many transitional forms. The spleen is small and of relatively minor importance. Apparently the kidney is the chief hemopoietic organ of the tadpole. At some time in the later developmental history of the frog, therefore, the locus of hemopoiesis becomes shifted from the kidney to the spleen. This is a clear case of what may be termed successive hemopoietic dimorphism.

It occurred to the writers that it might be of interest to find out whether the hemopoietic locus could be rapidly shifted experimentally by treating tadpoles with thyroid extract and thus inducing metamorphosis. The experiments performed to test this point have also thrown some light upon the part played by blood cells in metamorphic differentiation. In spite of the large number of investigations within recent years in the field of amphibian metamorphosis, no study has been made of the part played in the process by blood cell formation

and distribution. The importance of such a study is apparent when consideration is given to the fact that metamorphosis is fundamentally related to a change in metabolic rate, and must, therefore, involve hemopoiesis. Our experiments also indicate some features of the thyroid mechanism, some links in the complicated chain of reactions initiated by the thyroid secretion, by which its profound effects are accomplished in various regions of the body. Finally, the results corroborate previous conclusions of the writers in regard to the functions of the lymphocyte.

Material and Methods.

Bullfrog tadpoles (*Rana catesbeiana*) were used for the experiments. The first batch, fourteen in number, was collected early in January near the University of Virginia, and the second batch, twenty-two in number, on February 20th. These were judged to be about 9 or 10 months old. They averaged from 6 to 7 cm. in length, and possessed small insignificant hind legs.

Metamorphosis was initiated by feeding thyroid extract of sheep. A dry tablet of the extract was softened with water and small fragments inserted into the mouth of the tadpole on the tip of forceps. Control specimens were kept in a separate aquarium and killed at intervals throughout the course of the experiments. The tissues were in all cases fixed in the formalin-Zenker mixture of Helly and stained by the eosin-azure method of Giemsa.

The results given in this paper are based chiefly upon these thirty-six animals. In addition, however, observations have been made on fifty-three other tadpoles of greatly varying sizes collected during the months of March, April, and May.

EXPERIMENTS AND OBSERVATIONS.

In the first series of experiments metamorphosis was initiated in nine tadpoles. These either died or were killed, at intervals varying from 4 to 14 days after the first thyroid treatment. Normal controls were also examined. In the second series of experiments metamorphosis was initiated in ten tadpoles. These also either died or were killed, at intervals varying from 2 to 14 days after the first thyroid administration. Normal controls were also killed. Microscopic study was made of the tissues of these animals, including kidney, spleen, intestine, mesentery, fore and hind limbs, axillary lymph nodule, liver, thymus, head region, skin, and tail.

Attempts were also made to bring about complete metamorphosis. With one exception, these were unsuccessful, although some of the

animals lived for more than a month. Metamorphosis proceeded rapidly at first, but after about 10 days or 2 weeks usually slowed up, and the specimens died in the resulting more or less quiescent condition of "metamorphic stasis."

External Changes.

The early changes in the tadpole following thyroid administration are well known. Externally the most conspicuous changes are tremendous increase in vascularity and rapid growth of the hind limbs, shrinkage in body size due principally to the extrusion of the intestinal contents and to the shortening of the intestine, absorption of the tail, appearance of the fore limbs (the left fore limb always appearing first), and the gradual change from the tadpole type of face to the frog type.

Internal Changes.

A. The Kidney.—The kidney becomes smaller and somewhat paler. Upon section it is found to contain fewer blood cells. In the normal tadpole kidney the intertubular regions contain many blood cells, including both adult types of erythrocytes and leucocytes and those in their developmental stages (Fig. 1). After thyroid treatment, however, the intertubular regions become partially drained of their blood cells (Fig. 2). Although there is less hemopoietic tissue present in the kidney after thyroid treatment, it is nevertheless quite active. Many lymphocytes are in process of amitotic division, and in addition some mitotic figures may be seen. Lymphocytes, special granulocytes (neutrophils), eosinophils, together with transitional stages between lymphocytes and the eosinophilic and special granulocytes, are quite numerous. Transitional stages between lymphoid hemoblasts and erythrocytes may also be found. In no case is there total drainage or exhaustion of the hemopoietic capacity of the kidney.

B. The Spleen.—The spleen is somewhat variable in its response to thyroid treatment. In some animals in which metamorphosis proceeded rather slowly, the spleen was somewhat larger than normal, and its hemopoietic activity slightly increased. There is some evidence that mesenchymal cells in the nearby mesentery differentiate into lymphocytes, migrate to the spleen and become incorporated in it, thus contributing to its growth. In most of the animals, however, the spleen does not increase in size. It remains approximately the normal size, or becomes smaller, with its erythropoietic activity greatly heightened.

The chief function of the normal tadpole spleen is the production of erythrocytes from lymphoid hemoblasts. These, together with the cells of the splenic reticulum, are the predominating types of cells (Fig. 3). Thyroid administration markedly stimulates the differentiation of erythrocytes from lymphocytes. 2 days after the first feeding the spleen is characterized by the presence of large numbers of young erythrocytes, together with many intermediate stages between

these and their lymphocyte progenitors. In later stages the erythrocytes are carried off by the blood vessels, so that the percentage of lymphocytes to erythrocytes in the spleen increases. In those animals that reach a condition of metamorphic stasis, the spleen becomes partially or totally exhausted of its hemopoietic content (Fig. 4). Both lymphocytes and erythrocytes are drained from the spleen to be taken into the general circulation. These cells are not replaced rapidly enough to meet the demand set up by the increased metabolic rate induced by the thyroid treatment.

C. The Tail and Intestine (*Regions of Regressive Change*).—Following thyroid treatment there is an increase in the leucocyte content of the tail and of the intestine. The predominating types of leucocytes in the tail region are the special granulocytes, lymphoid phagocytes (phagocytes derived from lymphocytes), and to a less extent, lymphocytes and eosinophils. The special granulocytes contain no particles of cellular debris and perform little, if any, true phagocytosis. Tissues undergoing degeneration are removed by the lymphoid phagocytes. The special granulocytes may go to pieces and then be themselves ingested by the phagocytes. Apparently the occasional eosinophils in the tail region have a like history. The lymphocytes in the tail are possibly about to differentiate into phagocytes.

The intestine of a thyroid-treated tadpole is characterized by the presence in the mucosa of many eosinophils and lymphoid phagocytes. Some quiescent lymphocytes are also in evidence. There is plainly a tremendous loss of cellular elements by process of phagocytosis and by process of sloughing off into the intestinal lumen. This latter point has been recently stressed by Kuntz (1922). Eosinophils do not perform phagocytosis here, but in many cases appear to go to pieces in the lining of the intestine and are then ingested by the phagocytes. The complete absence of special granulocytes is striking.

D. The Limbs (*Regions of Progressive Change*).—Very conspicuous is the increased vascularization of the limbs a few days after the first thyroid feeding. Outside of the blood vessels the predominating type of leucocyte is the lymphocyte, and it is, furthermore, practically the only type of blood cell present here. In the normal tadpole there is a region of mesenchymal cells beneath the epidermis (Fig. 5). After thyroid treatment, however, a rather definite and continuous layer of lymphocytes appears here (Fig. 6). Some of these lymphocytes may come from the general circulation but the appearances suggest rather that they are mostly differentiated *in situ* from the mesenchymal cells. Occasional transition stages between mesenchymal cells and lymphocytes can be seen.

E. Other Regions.—The skin of the ventral, lateral, and dorsal trunk region, of a thyroid-treated animal which showed this lymphocyte layer in the rapidly growing limbs, was found to contain no similar subepidermal layer of differentiating lymphocytes. Examination was also made of the thymus, liver, minute axillary lymph nodule, and the head region, but the findings are not pertinent to our present interests and may be omitted in this paper.

INTERPRETATION AND DISCUSSION.

Thyroid administration raises the metabolic rate of the larval frog. This brings about profound changes in the larval hemopoietic organization and in the blood cell distribution. Our findings indicate that (1) an increased demand is set up for erythrocytes to furnish the physical basis for the maintenance of the new high oxidative level. Erythropoiesis is, therefore, stimulated in both spleen and kidney, especially in the former. (2) Increased metabolic rate involves also a demand for leucocytes, (*a*) for granulocytes and phagocytes to aid in the processes of regressive change, (*b*) for lymphoid hemoblasts which are the progenitors of erythrocytes as well as of granulocytes, (*c*) for lymphocytes to aid in stimulating growth of adjacent tissues in regions of progressive change. These matters will now be discussed more fully.

The demand for blood cells of all kinds is followed by partial drainage of erythrocytes and leucocytes from the kidney and spleen, and by the stimulation of both of these organs to increased hemopoietic activity. The kidney now produces chiefly leucocytes, the spleen erythrocytes. At this new high metabolic rate the spleen becomes a much more important organ. If the hemopoietic reserve is not drained too rapidly by excessive thyroid feeding, the spleen may be slowly stimulated to an increase in size in spite of the demands being made upon it. In animals treated in this way it may be said that partial drainage of the kidney, together with stimulation of the spleen, indicates that the shift of hemopoietic locus from the kidney to the spleen has been initiated. In no case so far have we been able to carry the shift to completion experimentally although some of our animals have been fed thyroid for more than a month.

Most of our thyroid-treated animals have died from 10 to 30 days after the first feeding. The initial rapid metamorphic differentiation progressively becomes slower until a condition of metamorphic stasis is reached, death usually occurring in this stage. The fundamental cause of death we believe to be anemia. The spleen of a tadpole in true metamorphic stasis is practically exhausted of its hemopoietic capacity. The erythrocytes are first drawn from the spleen, then the lymphoid hemoblasts are stimulated to differentiate into erythrocytes, and the latter are then in turn taken up by the general circula-

tion. Lymphocytes as such may also be drawn from the spleen. The splenic reticular cells may round up to differentiate into lymphocytes, and other lymphocytes may be brought into the spleen from other parts of the body. In any event, if the demand for blood cells is too great, the spleen is ultimately exhausted of its hemopoietic content and metamorphic stasis results, associated with a condition of anemia. Upon the success of the organism in meeting the tremendous hemopoietic demands of thyroid-accelerated metamorphosis, depends whether or not metamorphosis will be successfully completed. This conclusion does not preclude the possibility that metamorphosis may also be inhibited by other factors, such as the lack of proper coordination between the thyroid and the hypophysis.

In an earlier series of experiments upon adult frogs we have induced a marked increase in the hemopoietic rate by means of experimental hemorrhage, by repeated aspiration of blood directly from the heart (Jordan and Speidel, 1923). The results have shown that the ability of the frog to withstand successfully such treatment depends upon the ability of the lymphocytes, as progenitors of both leucocytes and erythrocytes, to form, to proliferate, and to differentiate rapidly enough into blood cells to meet the demands of the experiment. A similar result was obtained with the injection of saponin, a hemolytic toxin. Spleens totally or partially exhausted of their hemopoietic content were obtained in these cases. In the light of these experiments upon adult frogs, the writers were able to predict, before making any microscopic examination, that the spleen of a tadpole which had died in metamorphic stasis would show exhaustion of its hemopoietic content.

It is of interest in this connection to note that Swingle (1923) has attempted to bring tadpoles through this stage of metamorphic stasis by treatment with both thyroid and hypophysis transplants. He finds the response variable. In a few of the animals he believes the treatment is efficacious, in others not. It is difficult for the writers to see how the addition of the active principle of the hypophysis could help an anemic tadpole over the condition of metamorphic stasis unless it stimulated the production of more blood cells, a function which has never been ascribed to the hypophysis. It is probable that the tadpoles which were helped over this condition still possessed some hemopoietic

reserve. The writers do not mean to ignore the fact that metamorphosis is an extremely complicated process, the successful completion of which requires proper interaction between thyroid and hypophysis, and possibly other tissues. For the interaction to bring about the completed metamorphosis, however, it is also necessary that there be an adequate hemopoietic basis.

The phagocytic action of leucocytes in certain regions during metamorphosis is well known. Large mononuclear phagocytes derived from lymphocytes do most of the actual work of ingestion and removal of disintegrating cellular debris in both tail and intestine. In the intestinal mucosa are found many eosinophils, mostly gathered near the basement membrane of the epithelial lining. Our study of these cells suggests that they give off substances, often themselves going to pieces in the process, that aid in causing disintegration or other regressive changes of nearby tissues. They perform no phagocytosis, but may themselves be taken up later and removed by the lymphoid phagocytes. Kuntz (1922) points out that much of the intestinal lining is finally removed by being extruded into the lumen and passed out with the feces. The granulocytes in the tail region also appear to play no important part in the actual ingestion and phagocytosis of degenerating cells, but rather aid in breaking down surrounding tissues, thus rendering them more easy of ingestion and phagocytosis by the phagocytes. In brief, the granulocytes, both special and eosinophilic, appear to be mobile, unicelluar glands which give off substances that have a lytic effect on tissues undergoing regressive change. The writers do not hold that the secretions of these cells are the first cause of degeneration in these regions. As Morse (1918) has pointed out, a spontaneous breaking down of tissues occurs before mobilization of the granulocytes. Whatever initiates the degeneration process, however, the granulocytes aid in furthering it.

That certain leucocytes have in addition a growth-promoting function in certain regions of the tadpole during metamorphosis is also probable. Carrel (1922) found in the case of the chick that either leucocyte extract, or fluid in which leucocytes have been living for some time, when added to embryonic heart or fibroblast tissue cultivated *in vitro*, stimulated 100 per cent greater growth and differentiation than occurred in the control cultures. He concludes that leuco-

cytes, whether living or dead, give off substances which stimulate adjacent resting cells to grow and differentiate. We believe that the rapid growth of the limbs in thyroid-treated tadpoles may be partly ascribed to the stimulating effect of lymphocytes. Carrel's experiments dealt with all leucocytes taken together, a mixed culture. In the involuting tadpole there is a specific differential distribution of leucocytes, the lymphocyte being easily the predominant type in the limbs (Fig. 6). It is obvious, therefore, that any leucocytic secretion in this region must come from the lymphocytes. We suggest, accordingly, that the growth-promoting function of leucocytes, as demonstrated by Carrel, is due principally, if not entirely, to the lymphocyte component of leucocytes. Again, the writers do not hold that the lymphocytic secretion is necessarily the first cause of the growth effect in the limbs. But whatever initiates the growth reaction, the lymphocytes apparently aid in maintaining it. From the negative standpoint, it is hardly probable that either the granulocytes or the phagocytes, which are found in abundance in regions of regressive change, also give off growth-promoting substances.

This conception that the lymphocyte, in addition to its other functions, is a mobile unicellular gland with the function of stimulating growth of nearby cells need not be a surprising one. The lymphocyte is an embryonic type of cell, relatively undifferentiated. As such it is an element with pluripotential capacities. It is a well known fact that adult resting cells, when cultivated *in vitro*, are stimulated to grow when embryonic juice is added to the culture. The lymphocyte may be regarded as an embryonic relic that persists throughout life, a cell that is on hand at all times to bring stimulation (to contribute "embryonic juice") to any region where stimulation is needed.

The criticism may be made that, since the tadpole is an embryo, the limbs contain a supply of embryonic juice derived from the muscles, mesenchyme, etc., which should furnish the stimulation for growth. The answer to this is that the tadpole limbs, in the stages used in our experiments, are in a comparatively inactive condition and remain so for a considerable period of time. The larval bullfrog takes from 2 to 3 years to arrive at the period of metamorphosis. Histologically, the structure of the skin and connective tissue of the hind limb suggests a rather stable condition, approximating an adult type. These tissues, therefore, resemble adult tissues rather than embryonic.

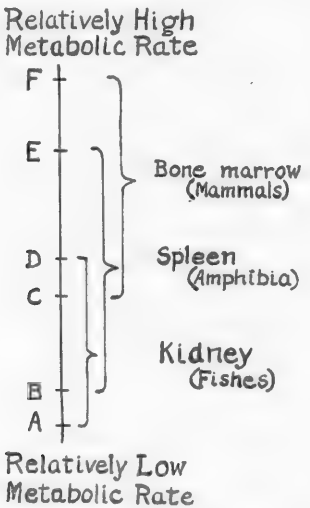
The conception of the lymphocyte as a growth-promoting cell leads to a consideration of lymphoid tissue in relation to growth. Scammon (1921) has constructed a curve showing the age variation in growth and size of lymphoid organs. These attain their greatest size and weight in man at about the age of puberty. It is interesting to note that this is also the period of considerable stimulation to new growth of many parts of the body.

In both embryonic and adult developmental changes it is known that successive "waves of growth" occur in the various organs and parts of an animal. Just as the lymphocytes aid in growth promotion of the limb tissues of a tadpole in metamorphosis, so they may be a part of the mechanism for bringing about and maintaining the less conspicuous increase in growth rate that occurs in vertebrates at definite periods in various organs and parts of the body.

Our findings are readily harmonized with Huxley's (1922, 1923) general conception of the mechanism of metamorphosis. The initiation of the shift of hemopoietic locus from the kidney to the spleen represents merely one aspect of the change from larval organization to adult organization, like the change from gills to lungs, or from the fin and tail method of locomotion to that by means of limbs. The larval hemopoietic organ, the kidney, is chiefly a leucocyte producer and functions best at the lower metabolic level. With the onset of metamorphosis its cells are largely drained and not adequately replaced. The adult hemopoietic organ, the spleen, is chiefly an erythrocyte producer and functions best at the higher metabolic rate. It is important to note, too, that at the higher metabolic rate there is a greater stimulation of erythropoiesis, a condition which probably explains why the spleen becomes the chief adult organ of hemopoiesis.

In the warm-blooded vertebrates the spleen is superseded as erythrocyte producer, this function being taken over by the red bone marrow. In these forms the metabolic rate is much higher than in the amphibia, and it is concluded that the bone marrow is here better adapted for erythrocyte production at the higher metabolic rate than is the spleen. In agreement with this idea is the observation that red bone marrow appears in the frog (*Rana pipiens*) in the early spring, after the hibernation period, at the time when there is great demand for new blood to support the rapidly increasing metabolic rate associated with the

breeding process. The life history of the frog, therefore, with respect to hemopoietic centers, presents a case of successive hemopoietic trimorphism. This is correlated to some extent, at least, with changes in the rate of metabolism. As shown in the diagram (Text-fig. 1), the kidney functions best at relatively low metabolic levels (*A* to *D*), the spleen at medium levels (*B* to *E*), and the bone marrow at high levels (*C* to *F*). Kidney and spleen may function together at a certain



TEXT-FIG. 1. Diagram to show the relation between a successive trimorphism of hemopoietic centers and rate of metabolism. *A* to *D*, range of metabolic rate at which the kidney functions as a hemopoietic center. *B* to *E*, range of metabolic rate at which the spleen functions as a hemopoietic center. *C* to *F*, range of metabolic rate at which the bone marrow functions as a hemopoietic center.

range of metabolic levels, as *B* to *E*. This condition occurs in the tadpole. Spleen and red bone marrow may be active at the same time at the range of metabolic levels, *C* to *E*, a condition that may be found in the leopard frog for a brief period in the spring. Or even all three organs may be active at a certain level, *C* to *D*. This last condition was found in an adult bullfrog examined in the spring. The spleen was most active, the bone marrow next, and the kidney least. A

similar condition was found in one of our thyroid-treated tadpoles, an animal which completed its metamorphosis and died 33 days after the first feeding.

Our comparative studies indicate that there is also a parallel phylogenetic trimorphism of hemopoietic centers as we pass up the vertebrate series. The chief hemopoietic organs in teleost fishes are kidney and spleen; in anuran amphibia, kidney, spleen, and transient red bone marrow; in birds and mammals, red bone marrow, lymph nodes, and spleen. The order seems to be, (1) kidney, (2) spleen, (3) red bone marrow. Correlated with this seriation in a general way, perhaps, is the fact that the thyroid, a regulator of metabolic rate, is of relatively small size in fishes, of medium size in amphibia, and largest in birds and mammals.

SUMMARY.

1. Thyroid-accelerated metamorphosis in the larval frog is accompanied by changes in the hemopoietic centers and in the blood cell distribution in the various regions of the body. These changes are interpreted as results of the fundamental change in basal metabolic rate induced by the thyroid treatment.

2. There is initiation of the shift of hemopoietic locus from the kidney, the larval hemopoietic organ, to the spleen, the adult hemopoietic organ. The spleen, being chiefly an erythrocyte producer, becomes of greater importance with the transition from the lower metabolic rate to the higher, since greater erythropoiesis becomes necessary to supply the physical basis for the maintenance of the higher metabolic rate.

3. It is suggested that the appearance of red bone marrow in the later history of the frog is correlated with a still higher metabolic rate. Phylogenetically, in the vertebrate series, red bone marrow is also associated with higher metabolic rate.

4. The new metabolic rate initiated in tadpoles by thyroid administration sets up a demand for (*a*) erythrocytes, (*b*) granulocytes and lymphoid phagocytes for distribution to regions of regressive change, (*c*) lymphocytes, (1) as progenitors of erythrocytes, granulocytes and phagocytes, (2) for promoting growth of cells in regions of progressive change.

5. Upon the hemopoietic reserve, which in the last analysis is the lymphocyte (and its mesenchymal precursor), depends the extent to which metamorphosis will proceed. Inability on the part of the hemopoietic centers, chiefly the spleen, to keep pace with the demand for blood cells during metamorphosis results in metamorphic stasis, a condition of anemia which is usually followed by death.

6. The growth-promoting function of leucocytes, as demonstrated by Carrel, is probably to be ascribed to the lymphocyte component of leucocytes.

7. The granulocytes have probably also a glandular function, and may exert a lytic effect upon adjacent tissues in regions of regressive change.

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EXPLANATION OF PLATES.

PLATE 34.

FIG. 1. Longitudinal section of kidney of normal bullfrog tadpole. Parts of three tubules may be seen. The intertubular region is filled with many erythrocytes, leucocytes, and transitional forms. × about 500.

FIG. 2. Longitudinal section of kidney of thyroid-treated tadpole, killed 4 days after the first feeding. The intertubular regions are largely drained of blood cells. (This area somewhat exaggerates the average amount of drainage.) × about 500.

PLATE 35.

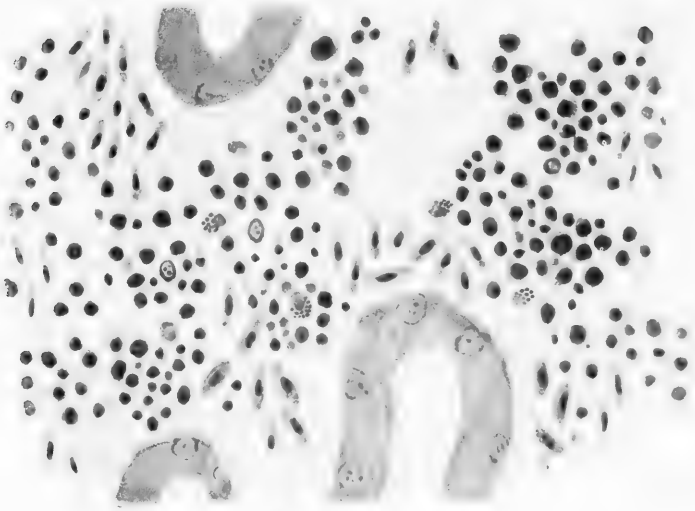
FIG. 3. Cross-section of spleen of normal tadpole. The principal cellular constituents are erythrocytes, lymphocytes, and splenic reticular cells. × 1,000.

FIG. 4. Cross-section of spleen of a thyroid-treated tadpole that died in metamorphic stasis. The spleen is almost totally drained of its hemopoietic content. × 1,000.

PLATE 36.

FIG. 5. Vertical section of skin of hind limb of a normal tadpole showing epidermis, *e*, and dermis, *d*. In the dermis may be seen mesenchymal and connective tissue cells and fibers. Note the stable, definitive, histological appearance of this skin. × 1,000.

FIG. 6. Vertical section of skin of hind limb of a thyroid-treated tadpole in which rapid growth is taking place. In the dermis, *d*, just beneath the epidermis, *e*, may be seen a conspicuous layer of lymphocytes, *l*. × 1,000.



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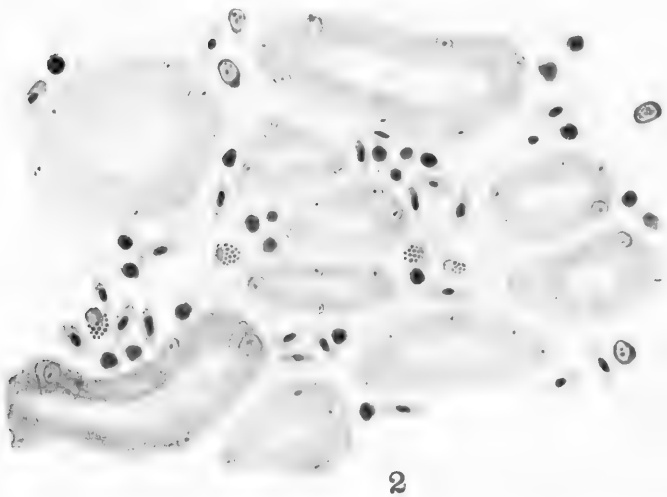

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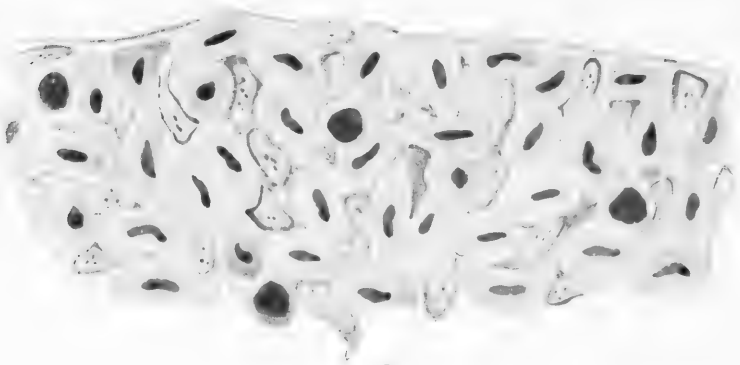
(Jordan and Speidel: Blood cell formation and distribution.)



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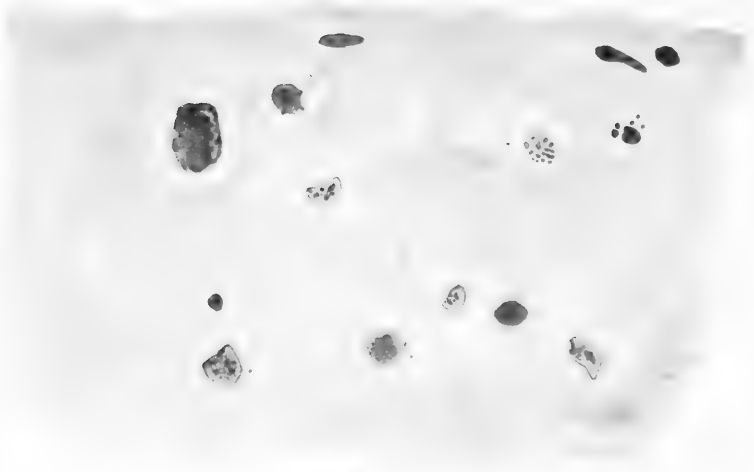

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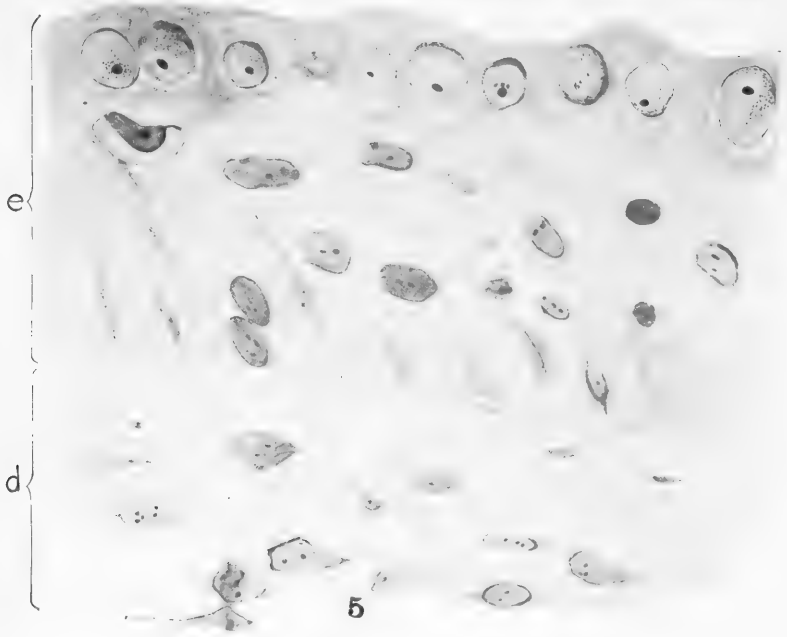

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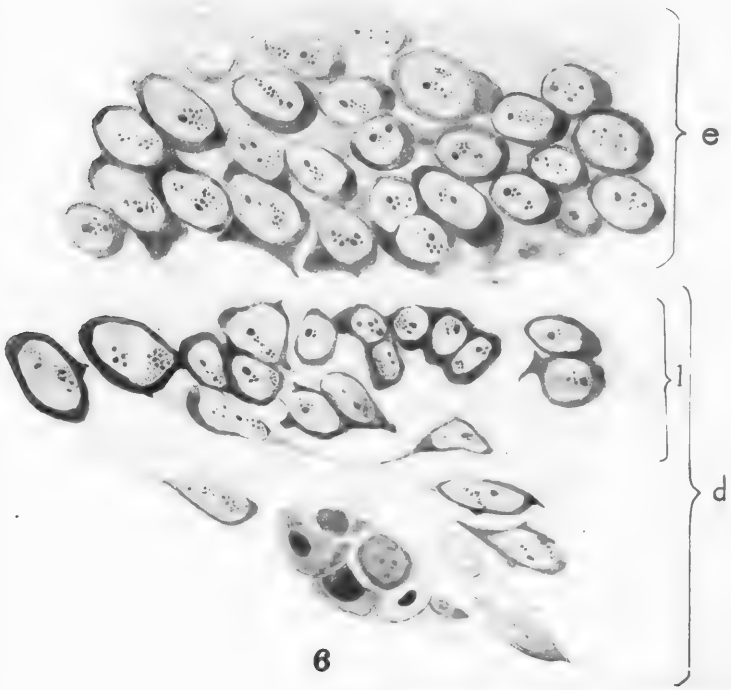

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(Jordan and Speidel: Blood cell formation and distribution.)

THE INFLUENCE OF THE ADRENAL GLANDS ON RESISTANCE.

I. The Susceptibility of Adrenalectomized Rats to Morphine.

By W. J. M. SCOTT,* M.D.

(*From the Laboratory of Surgical Research, Harvard Medical School, Boston.*)

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One of the early theories in regard to the function of the adrenal gland was that it acted as a detoxifying agent (1, *a*). While no convincing proof of this hypothesis was presented, many scattered facts have been brought out which show that the adrenals are involved in the reaction of the organism to infection and intoxication, and which suggest that these glands play an important rôle in the protective mechanism of the organism. In severe infections and intoxications pathological changes in the glands occur regularly, varying in intensity from congestion and edema to hemorrhages and foci of necrosis (2, 3) and the characteristic lipoids in the cortex show striking alterations in distribution and amount (4, 5). Chronic infections and intoxications in animals are accompanied by a marked hypertrophy of the cortex (6, 7). These effects of acute and chronic infections and intoxications on the adrenals invite the consideration of the influence of these glands on the resistance to such conditions.

In addition to the pathological changes mentioned, the early literature dealing with this relationship of the adrenal confines itself chiefly to three lines of argument: (1, *b*) the resemblance of the effects of adrenal ablation to a progressive intoxication (1); (2) the toxic properties of blood and tissue extracts from adrenalectomized animals, and conversely the protective properties of normal blood in adrenalectomized animals (8, 9, 10); and (3) the protection afforded by mixtures of the adrenal gland or its extracts with various toxic agents (11, 12).

* Arthur Tracy Cabot Fellow, in charge of the Laboratory of Surgical Research, Harvard Medical School, Boston.

None of these points is supported by convincing evidence of a detoxifying function of the adrenal. Entirely non-specific changes explain most of the phenomena under the third heading (13).

The direct approach to the problem by determining the resistance of surviving adrenalectomized animals has been attempted by several investigators. Most of the earlier reports were unsatisfactory due to the lack of sufficient control (unsatisfactory methods, no operative control animals, etc.) In 1921, however, Lewis (14) studied the resistance of adrenalectomized rats to several drugs in a comprehensive and adequately controlled manner. After reviewing the previous results with this method, he reported a definite increase in the susceptibility of rats after adrenalectomy to a number of different drugs. Morphine gave the most notable result, in that $\frac{1}{400}$ of the minimal lethal dose was sufficient to kill adrenalectomized rats (15). Stewart and Rogoff were unable to confirm these results (16).

This type of reaction to adrenal insufficiency deserves careful study on account of its importance in throwing light on the functions of the adrenal glands, and especially in regard to the involvement of the adrenals in infections. It is, therefore, the purpose of this paper to report the effect of adrenalectomy on the susceptibility of rats to morphine. This is undertaken as a preliminary to the investigation of the influence of the adrenal glands on the resistance to infections and intoxications.

The adrenal gland in mammalia is an organ composed of two separate tissues, cortex and medulla, entirely distinct in origin, morphology, and cellular chemistry. No definite correlation in the function of these two tissues has been proven. In certain lower species they exist as separate organs (1, *c*). The variations in the resistance of adrenalectomized rats to morphine observed in this study are believed to be entirely dependent on the removal of cortical tissue, and not related to epinephrine. This point will be discussed later.

Methods.

Experimental Animal.—The choice of the appropriate experimental animal is a very important matter in all work with the adrenals on account of the significant variations in these glands in the different species. The best method so far re-

ported for producing an adrenal insufficiency giving symptoms, without causing death, is by adrenalectomy in a series of animals possessing adrenal accessories (17). The rat and rabbit are the only common adult laboratory animals of which a large proportion possess cortical accessory tissue. On this account a large number of these two species will survive double adrenalectomy, a fact amply attested by the confusion which it caused for many years in demonstrating that adrenal tissue is essential to vertebrate life. The survival of adrenalectomized rats was not explained until it was shown that this animal usually possessed numerous accessory cortical rests far removed from the adrenal gland, chiefly in the vicinity of the sex glands, and microscopic in size (18). For reasons of economy of materials and work the rat is much to be preferred in such a study, where a relatively large number of animals are required. Stock rats of the Bussey Institute, *Mus norvegicus*, predominantly white with some color admixture, were used.

Procedure.—Rats under constant and identical laboratory conditions were observed for several days. Then at the same session six to twelve rats were operated upon alternately by one of two procedures, of which the first was double adrenalectomy, and the second was some control operation producing similar traumatization. This usually consisted in single adrenalectomy with some manipulation on the opposite side which left that adrenal and its blood supply intact. 7 days later, those rats that were fully recovered, eating well, and indistinguishable in behavior from normal rats, were injected with morphine sulfate.

It became evident early in this work that certain factors, not absolutely necessary with normal animals, were essential in the handling of adrenalectomized rats. All animals were kept in separate cages before and after operation, the room temperature was kept constant at 75–78°F., and they were on a completely adequate diet for several days before operation and continuing throughout the experiment. The diet used was kindly furnished us by Dr. Reid Hunt and was of the following formula.

Rolled oats	15 parts
Hominy	60 "
Dried meat scrap	14 "
Dried milk powder	10 "
Salt	1 "

15 gm. of this mixture were given the rat each day, and that remaining from the day before was weighed to calculate the amount eaten. The individual cages had two compartments, one containing the food-dish and water-tube, the other, paper shaving bedding. Water was constantly available.

A very good judgment as to the condition of the rat can be formed by an estimate based upon the following four factors: (1) food intake, (2) weight variation, (3) character of stool, and (4) behavior. The measurement of food intake

before operation is valuable as determining the individual base line. Normally the rats eat 10 to 15 gm. per day, though a reduced amount for any one day is without significance.

Diminished appetite is one of the most important signs of illness in rats. Any change in weight taken in conjunction with the food intake offers a clue as to the metabolic condition of the animal. On the diet specified the stools are invariably firm in the controls, while diarrhea develops in some of the adrenalectomized rats. As to behavior, the animals used were all active, invariably reacted definitely to blowing (though to quite different degrees), and kept their eyes open after being aroused; while the deviation from this behavior in the experimental animals varied from inactivity and loss of appetite to coma. A very striking picture was occasionally presented as a most severe reaction, either after operation or injection; *i.e.*, an adrenalectomized rat losing weight rapidly, with a persistent diarrhea, eating little or nothing, sitting very quietly in a hunched-up position, fur ruffled, eyes nearly closed, and not responding at all to gentle blowing.

Operation.—Adrenalectomy in rats has been repeatedly performed by previous investigators. The lumbar or back route with incisions through the abdominal wall on both sides has been employed (19, 20). It was found at the beginning of this work that a single midline ventral incision was as satisfactory and more expeditious for bilateral adrenalectomy. Ether anesthesia was always used, by the open method after induction. The operative field was prepared by shaving and bichloride sponges. The incision, 2 cm. in length, begins just below the xiphoid. After opening the abdomen a nasal speculum is carried between the lower lobe of the liver and the right kidney, and on opening the blades the right adrenal is exposed as a pinkish yellow body 1½ to 4 mm. in diameter, embedded in a small fat strip running along the vena cava. With a double ring-curret-bladed biting forceps the edge of which has been dulled the adrenal is now picked up. (The adrenal slips into the opening of the rings and is not crushed.) A small curved clamp is put on the pedicle, and by traction on the forceps, while the clamp is held rigid, the adrenal is pulled from its bed. Bleeding may be controlled by a ligature or clip around the pedicle which is caught by the clamp, but usually a sponge tip carried to this point and held with gentle pressure will control all bleeding after removing the clamp. The capsule is usually intact. Care must be exercised in putting the clamp on the pedicle not to catch the vena cava or renal vein, and any traction on this forceps is apt to tear the vena cava. Usually the right adrenal in the rat is far enough from the vena cava to be removed in this way without much difficulty. This anatomical relationship is another important advantage of using rats rather than rabbits, since the vena cava wall has frequently to be partially ligated in removing the rabbit's right adrenal.

The removal of the left adrenal of the rat is even more simple than the right. The spleen is caught by its tip and delivered out of the wound. With the nasal speculum opened between the left kidney and the mesenteric attachment of the spleen the left adrenal is exposed, embedded in a rather moveable mass of fat.

It is removed in the same manner as the right, the spleen returned to the abdomen, and the wound closed in two layers with silk. At no time are the intestines deviscerated. The whole operation usually requires only 10 minutes; within another 15 minutes the animal is walking about its cage.

That shock does not play an important rôle in the outcome is shown by an analysis of the first 100 rats operated upon in this way, 58 of which had double adrenalectomy, and 42 single adrenalectomy. Only three of these animals died within 3 days; two of the doubly and one of the singly adrenalectomized, and in each of these cases a severe hemorrhage was responsible. In two of the first fifteen adrenalectomies, the capsule was broken at one point and a small piece of it left. The cortical cells rapidly undergo compensatory hypertrophy under such circumstances. In none of the later cases has a fragment of the gland been found at autopsy.

Presentation of Data.

Results of Adrenalectomy in Rats.

On the basis of the results of adrenalectomy in the first fifteen rats, the precautions previously mentioned as being important in the care of such animals were evolved. This original group underwent approximately the same operative procedures as later groups, but they were kept before and after operation in stock cages each containing several rats, and on a varying diet consisting chiefly of oats with bread, milk, and cooked meat scrap. Under this regime they were in excellent condition before operation, yet of eleven doubly adrenalectomized rats seven died in from 3 to 9 days; four survived and seemed in good health at the end of a week. The four singly adrenalectomized survived without symptoms.

The standard balanced diet and individual cages were then introduced and in the next 46 rats, 25 were doubly adrenalectomized with only three deaths. This contrast is shown graphically in Table I. Whether the unbalanced diet or the segregation is responsible for the heavy mortality in the first group has not been worked out. However, that it is due to a metabolic disorder is suggested by the development of a severe diarrhea which persists to death although the animal is eating almost nothing and is put into a separate cage. The protocol of Rat 13 illustrates this type of reaction very well. In seven of the ten deaths of adrenal insufficiency definite hemorrhagic necrotic areas were found in the mucous membrane of the stomach.

At the end of a week after adrenalectomy, if the rat is active, eating well, and has not lost 10 per cent of its body weight, it will survive indefinitely under optimum conditions. But a certain number of such animals are still sensitive to conditions that they would have survived before operation. They may die if put back into stock cages.

TABLE I.

Mortality of Double Adrenalectomy in Rats.

	Total.	Survived.	Died.	Mortality.
				per cent
Stock group	11	4	7	64
Rats in individual cages on standard diet	25	22	3	12

Susceptibility to Morphine.

1. In Operative Controls.—Seventeen rats were used as operative controls. One adrenal was removed and some operative procedure carried out on the opposite side, so that the factors of the operation would be as nearly as possible identical with double adrenalectomy. The right adrenal was usually excised, as this is technically more difficult, and is occasionally accompanied by hemorrhage. On the left side the procedure usually consisted in the excision of a piece of fat in the immediate vicinity of the adrenal gland; sometimes the capsule was stripped from the upper pole of the kidney. If a hemorrhage was encountered in excising the first adrenal, that rat was used as a control animal and the second adrenal was not removed. A small dose of morphine was purposely chosen in order that minor variations in susceptibility dependent on such factors as hemorrhage, appetite, etc., would not appear in the results. Of these seventeen operative controls fourteen survived doses ranging from 50 to 100 mg. per kg., and three died, a mortality of 18 per cent. (Table II shows the exact doses given.) The fatal cases were interesting; one was pregnant, the second had some obscure pathological condition with periportal changes in the liver and hemorrhages into the remaining adrenal, and no reason for the fatal termination in the third was apparent.

2. In Doubly Adrenalectomized Animals.—Twenty-two doubly adrenalectomized rats were injected with morphine sulfate subcutane-

ously in doses ranging from 10 to 30 mg. per kg., the maximum being $\frac{3}{5}$ of the minimum dose given the control rats. Fifteen of these rats were killed by such small amounts of morphine; seven survived, the mortality being 68 per cent (Table III). This effect of adrenal insufficiency is shown by a comparison of the different results of small doses in the adrenalectomized and control animals. Five of eight adrenalectomized rats were killed by $\frac{1}{10}$ of the minimum dose that was fatal to any of the operative control animals.

The three adrenalectomized rats that had survived 10 mg. per kg. were reinjected 11 days later (18 days after operation) with 30 mg.

TABLE II.

Resistance of Operative Controls.

Morphine sulfate, *mg. per kg.*	100	80	60	50	Totals.
Total No.	9	2	2	4	17
Survived	6	2	2	4	14
Died	3	0	0	0	3

TABLE III.

Resistance of Adrenalectomized Rats.

Morphine sulfate, *mg. per kg.*	30	20	10	Totals.
Total No.	7	7	8	22
Survived	3	1	3	7
Died	4	6	5	15

per kg. of morphine sulfate. Two of the three died, and one survived. As a matter of fact, then, of the twenty-two adrenalectomized rats tested, seventeen (77 per cent) were killed by morphine sulfate in amounts of 30 mg. per kg. or less. Only the results of the original injection are entered in Table III. Both adrenalectomized and control animals were usually injected 1 week after adrenalectomy; a few were tested in the 2nd week.

Only those adrenalectomized rats were used for this purpose that were active and eating well at the time of injection. However, it was apparent that many of the adrenalectomized animals did not eat well

for 2 or 3 days after operation, while the controls usually had regained a normal appetite within 24 hours. This fact was reflected in the average weight losses after operation. In the control rats it was 1 per cent, while in the group of adrenalectomized rats it was 4.6 per cent at the time of injection. Furthermore, the seven adrenalectomized rats that survived morphine lost only 2.7 per cent in weight, while the fifteen that were killed by morphine had an average weight loss of 5.3 per cent. This comparison suggested that the difference in food intake might be a factor in the decreased resistance. But this hypothesis seemed unlikely, as a few of the susceptible adrenalectomized rats ate well even immediately after operation, and an occasional one of the control animals had a poor appetite and lost weight, yet survived the larger doses of morphine. The lack of correlation between food intake and susceptibility of individual rats is illustrated by Protocols 2 and 3.

3. In Controls with Diminished Food Intake.—The effect of a diminished food intake was tested in the following manner. Operative control rats were prepared as in the previous experiments. They received no food for 48 hours after operation. The first group of three were then each given 2 gm. of food a day. 7 days after operation they had thus received a total of 10 gm. of food and had lost 28 per cent (average) of their pre-operative weight. They were given 50 mg. of morphine per kg. Two of the three survived this dose and one was killed by it. It was obvious from the weight loss and food intake that this was a much more drastic diminution in nutrition than any of the adrenalectomized rats had experienced.

Therefore, in the next group of four rats, they were fasted for 2 days after operation and then allowed 4 gm. of food apiece per day. On this ration the weight loss in each case was greater than the maximum (11 per cent) found in any of the adrenalectomized rats. 1 week after operation these four controls, whose weight loss averaged 15 per cent, were injected subcutaneously with 60 mg. of morphine sulfate per kg. (twice the maximum dose given any adrenalectomized animal). All survived this dose. Thus, of the seven animals with greatly diminished food intake, only one was killed by morphine, though given in much larger doses than to any of the adrenalectomized rats. It seems justifiable, then, to conclude that the decreased resistance of adrenalectomized rats is not due to a diminished food intake. (Table IV.)

4. By Intravenous Administration.—In order to eliminate any variation in absorption, four rats were injected intravenously with morphine sulfate solution. Two operative controls were given 45 mg. per kg., which was about $\frac{1}{2}$ of the minimum lethal dose for unoperated rats. Before the injection had been completed respiration had become imperceptible, and the rats were rigid when put back into their cages. Within a few minutes respiration became perceptible; the condition of the rats improved gradually. In 3 hours the coma had disappeared, and the rats were making voluntary movements, though they were still quiet. The following day they had completely recovered, were alert, active, and had eaten their full rations (15 gm.).

Two adrenalectomized rats were injected at the same time, one with 9, the other with $4\frac{1}{2}$ mg. per kg. ($\frac{1}{5}$ and $\frac{1}{10}$, respectively, of the dose given the operative controls). The immediate effect of these small

TABLE IV.

Resistance of Operative Controls with Diminished Food Intake.

	No. of rats.	Food intake per rat, 1 week.	Weight loss (average).	Morphine sulfate.	Survived.	Died.
		gm.	*per cent*	*mg. per kg.*		
	3	10	28	50	2	1
	4	20	15	60	4	0
Total.	7	10 to 20	15 to 28	50 to 60	6	1

doses was in sharp contrast to the complete prostration and respiratory paralysis of the control animals. Both rats became quiet, but did not stop breathing, and they made voluntary movements on stimulation. Instead, however, of showing evidence of recovery within 3 hours as the controls had done, their condition became worse, and both died.

From one of the operative controls that survived the morphine intravenously, the remaining adrenal was removed after an interval. A week after the second operation, being in excellent condition, it was injected intravenously with $\frac{1}{2}$ of the dose that it had formerly withstood. The immediate effect was less pronounced than previously, but the rat became progressively worse and died 36 hours later. In this one animal, then, is seen survival, with one adrenal intact, following a dose double that which proved fatal to it after the second adrenal

had been excised (Protocol 4). The same striking increase in susceptibility to morphine is demonstrated in adrenalectomized rats when the drug is administered intravenously.

TABLE V.

Comparative Resistance of Rats after Excision of One Adrenal and of the Second.

Rat No.	Weight at operation.	Adrenal excised.	Days between operation and injection.	Weight at injection.	Injection of morphine sulfate.	Result.	Remarks.
	gm.			*gm.*	*mg. per kg.*		
10	140	Right.*	7	144	100	Survived.	14 days between operations.
	145	Left.	6	152	10	"	Fragment of right adrenal not removed.
			15	180	20	"	
			25	175	100	"	
16	340	Right.	11	320	100	"	18 days between operations.
	327	Left.	7	320	10	"	
			10	305	50	Died.	
17	340	Right.	11	335	100	Survived.	18 " " "
	332	Left.	7	325	10	"	
			10	315	50	Died.	
18	250	Right.*	11	260	100	Survived.	18 " " "
	?	Left.	7	260	10	"	Fragment of right adrenal not removed.
			10	252	50	"	
19	320	Right.	10	305	100	"	17 days between operations.
	307	Left.	7	278	20	Died.	
22	402	Left.	10	379	100	Survived.	17 " " "
	362	Right.	7	367	30	Died.	
53	202	Right.	7	147*	50	Survived.	13 days between operations; only 10 gm. of food given in first week after first operation.
	180	Left.	7	185	30	"	
54	199	Right.	7	148*	50	"	13 days between operations; only 10 gm. of food given in first week after first operation.
	175	Left.	7	175	30	Died.	

* See under remarks.

5. In the Same Rats After Single and Double Adrenalectomy.—From eight operative control rats the second adrenal was removed and they were reinjected subcutaneously with smaller amounts of morphine. Six of them had survived 100 mg., and two 50 mg. per kg. of morphine

sulfate after one adrenal had been removed. After the second adrenal had been removed, five of the eight were killed by 50 mg. per kg. or less. Time intervals, weights, and exact doses of morphine are given in Table V. It is interesting to note that two of the three rats that survived had a fragment of adrenal left *in situ* at the first operation, as subsequently discovered.

Subacute Type of Reaction to Morphine.

In normal animals morphine given subcutaneously produces its effect within a short time, terminating in death or recovery. The outcome is decided within 24 hours, usually in much less time than this. Our operative control animals were alert, active and eating well on the day following the injection in almost every case. Morphine sulfate injected subcutaneously or intravenously was fatal to twenty-six rats doubly adrenalectomized in 1 or 2 stages. Twelve of these (46 per cent) died more than 24 hours after injection. The most striking instance of this reaction ocurred in Rat 24; though profoundly affected by the drug, it lived for 10 days after injection (Protocol 5). Others lived for 3 to 5 days. They never recovered their appetite or normal behavior. Some of the doubly adrenalectomized rats that survived their small doses of morphine were below par for 2 days after injection, while the control animals receiving two to ten times as much morphine were invariably normal in behavior 24 hours afterwards.

DISCUSSION.

In opposition to Stewart and Rogoff (16), the statement of Lewis (15) that rats are much more susceptible to morphine after adrenalectomy, has been confirmed in this study. The cause of the discrepancy seems apparent from Stewart and Rogoff's protocols. None of their rats was tested within a period of 6 weeks after operation whereas a marked hypertrophy of adrenal cortical cells will occur within 4 weeks. This fact is illustrated by one of our rats in which a piece of adrenal cortex, too small to be seen, was left at operation. 26 days later the hypertrophied fragment was found to be $\frac{2}{3}$ the size of the original gland. Wiesel (18) has shown that the adrenal accessory rests of cortical cells occurring in the neighborhood of the sex glands undergo compensatory hypertrophy.

The cause, it appears, of the increased susceptibility to morphine during a phase of adrenal insufficiency is not a local traumatization of structures in the neighborhood of the adrenal as our control operations and those of Lewis seem conclusively to show. It is also not due to a general, non-specific disturbance of the endocrine balance since Olds (21) found no increased susceptibility in thyroidectomized rats to morphine. It would seem that a decreased resistance to morphine is a definite effect of adrenal insufficiency, either directly or through some secondary reaction.

Hunt and Seidell (22) have demonstrated that feeding thyroid extract to rats increases as much as ten times their susceptibility to morphine. This suggests that the increased susceptibility of adrenalectomized rats might be due to a stimulation of thyroid secretion.

Marine and Baumann (23) have shown that thyroid function is necessary for the characteristic increased heat production of adrenal insufficiency in rabbits. Scott (24) has presented some evidence of a rapid depletion of thyroid colloid by partial adrenal insufficiency in cats. Both of these reports present indirect evidence of an autogenous increase in the thyroid hormone in certain types of adrenal insufficiency. However, there is an important difference in the reactions of adrenalectomized and of thyroid-fed rats to morphine.

In Hunt's study, 51 thyroid-fed rats were given about the minimum lethal dose (23 survived and 28 died); also 12 normal rats were reported (7 survived and 5 died). The susceptibility of the rats receiving thyroid was increased in varying degrees. In every instance, though, the result was determined in less than 24 hours. In the thirty-three fatal cases, all but two of the rats died within 12 hours (these lived 13 and 20 hours, respectively). Contrast with this the effect of morphine observed in adrenalectomized rats. Susceptibility is increased, but one-half of the fatalities occur from the 2nd to the 10th day after injection. In view of this dissimilarity, it seems improbable that increased thyroid function is chiefly responsible for the diminished resistance of adrenalectomized rats.

No attempt was made to determine the minimum dose that could be found to kill the most susceptible adrenalectomized rats. A relatively small number of the adrenalectomized rats are extremely sensitive. Substances have proven fatal to such that a majority of adrenalectomized animals survive. (This is probably due to the marked variation in the amount of cortical accessory tissue.) Therefore, the minimum lethal dose seems to be a less satisfactory method of comparison with adrenalectomized rats.

It was considered far more important for the purposes of this investigation to determine the type of reaction in a number of adrenalectomized rats. They were, therefore, given the drug in doses that were never fatal to the operative control animals. By this method one of the most instructive phenomena observed was quite unexpectedly discovered; namely, the fundamental disturbance of the adrenalectomized rat's condition lasting several days following the morphine injection, and usually terminating in the death of the animal (though evidence of it was also discernible in some of those that recovered). This type of reaction has not been emphasized by previous investigations, and is easily overlooked when the M.L.D. is used as the sole basis of comparison. It occurred only in adrenalectomized animals, and was never seen following single adrenalectomy, even in a partially fasting animal. This profound and unusual effect of morphine on the condition of adrenalectomized animals seems to us to be of great theoretical importance.

The decreased resistance in adrenal insufficiency could be caused by (1) failure to excrete or destroy the drug, and (2) increased susceptibility to a given concentration of the drug.

1. Failure to Rid the Body of the Drug.—Marshall and Davis (25) have shown that in the total adrenal deficiency of cats, even while the blood pressure is still maintained, the nitrogen waste products are excreted much more slowly, and accumulate in the blood. It is not known whether this is the case in the relative insufficiency of adrenalectomized rats (cortical accessories present). However, only a small proportion of the morphine is excreted by the kidneys, and nothing is known about the efficiency of the intestinal excretory function or about the rate of destruction of drugs in adrenal insufficiency. That the persistence of the drug is responsible seems unlikely, from the long period (10 days) that such animals may survive, and from the relatively small amount of the drug given.

2. Increased Susceptibility to a Given Concentration.—The conclusion that there is an increased susceptibility of the animal to a given concentration of the drug seems to be forced upon us. The mechanism of this is not clear. The subacute form of reaction to morphine in the adrenalectomized animal strikingly resembles the cases of fatal adrenal insufficiency seen in rats. Lack of appetite, marked loss of

weight and reactivity, terminal diarrhea, and hemorrhages into the gastric mucosa are usual in each of these conditions, though if the morphine-injected adrenalectomized rats had not been given the drug they would have lived indefinitely without symptoms. The similarity is sufficiently marked to suggest that both conditions are fundamentally related.

Final proof as to the nature of the mechanism whereby adrenal insufficiency affects the susceptibility of an animal is not available. But the most logical explanation of the known facts seems to us to be as follows: Insufficiency of adrenal function greatly diminishes some normal metabolic reserve factor. If this effect is too great the animal dies of adrenal insufficiency. Under optimum conditions, though, most rats show little abnormality. However, the effect of a harmful substance such as morphine is sufficient to overcome the diminished reserve factor. The subacute effect is seen when the reserve is used up by the reaction but not greatly exceeded. On this hypothesis the variation in the effect of a given amount of morphine is explained by the various degrees of adrenal insufficiency due to the different amounts of cortical accessory tissue in each rat. Lewis also explains the diminished resistance to certain drugs as a metabolic disturbance, which increases the sensitiveness of cells to toxic substances, and suggests that the mechanism of this may be a quantitative or qualitative change in their lipoid equilibrium (14).

No new evidence is here presented as to which part of the adrenal gland is essential in maintaining the resistance to morphine. The variations in resistance seem to be correlated with the status of the accessory adrenal rests which are made up of cortical cells. It seems improbable that the secretion of adrenin plays any part in the diminished resistance following adrenalectomy. However, further evidence on this point will be presented in a later paper.

The effect of diet in adrenal insufficiency is an important problem which should be carefully studied. McCarrison (26) has pointed out marked pathological changes in the adrenal glands due to unbalanced diets. A completely balanced, uniform diet was given our rats after the first few experiments. The results of these first fifteen adrenalectomies, however, suggest that animals in a state of partial adrenal insufficiency are susceptible to dietary factors that would produce no symptoms in normal animals.

The high mortality of adrenalectomy reported for rats in much of the older literature is undoubtedly due chiefly to the operative technique. Thus H. and A. Cristiani (27) report only three rats out of twenty-nine surviving adrenalectomy for more than 20 hours; and Schwarz (19) claims that removal of both adrenals at one operation is fatal. However, in rats the mortality of adrenalectomy may be high in spite of a perfectly adequate operative technique. Stewart and Rogoff (28) report eight fatalities in 1 to 14 days out of a group of thirteen rats. This corresponds closely with our results in the first fifteen adrenalectomies. By establishing (1) a carefully controlled standard diet, (2) isolation, and (3) uniform room temperature, our mortality immediately dropped from 64 to 12 per cent while the technique of operation was unchanged.

This study was undertaken to determine the types of reaction of adrenal-insufficient animals to the harmful effects of a drug. It is intended as an introduction to the investigation of the influence of adrenal function in bacterial intoxications.

CONCLUSIONS.

1. The majority of rats (*Mus norvegicus*), because of accessory cortical tissue, will survive double adrenalectomy indefinitely under optimum conditions.

2. Resistance to morphine is greatly diminished in healthy adrenalectomized rats tested before hypertrophy of the accessories occurs.

3. This greater sensitiveness seems to be due to some fundamental alteration in metabolism dependent on a partial adrenal insufficiency.

Protocol 1.—Rat 13; brown and white, male. Sept. 15, 1922. In stock. Sept. 18. Active, vigorous, and very vicious. Weight 281 gm. Operation, double adrenalectomy. Prompt recovery. Sept. 21. Very active and vicious. Sept. 22. Jumps viciously this morning on opening the cage. This afternoon, quiet and marked diarrhea. Transferred to individual cage. Eating nothing. Sept. 23. Diarrhea. Quiet. Sept. 24. Diarrhea diminished, more active, but is not vicious. Appetite poor, ate 5 gm. Sept. 25. Diarrhea, ate nothing. Sept. 26. Diarrhea still marked, ate nothing. Died this afternoon.

Autopsy.—Weight 215 gm. Thyroid, heart, and lungs normal. Abdomen: intra-abdominal fat almost completely gone. Adrenals absent. Stomach: definite multiple minute hemorrhages in the mucous membrane throughout the pyloric part. Intestines: several loops of small gut deep red in color. Liver: nutmeg color.

Protocol 2.—Rat 43; white, male. Oct. 20, Active. Weight, 236 gm. Operation, excision of right adrenal and excision of piece of pancreas near left adrenal.

Moderate bleeding. Oct. 22. Has not eaten since operation. Oct. 23. Active, beginning to eat. Oct. 27. Active, eating well now. Slight diarrhea. Weight, 222 gm. At 11:10 a.m. injected subcutaneously with 1.78 cc. of 1 per cent morphine sulfate solution (= 80 mg. per kg.). 1:30 p.m., quiet. 6 p.m., recovering. Oct. 28. Active, eating well. Nov. 10. In good condition. Sacrificed Jan. 8. Right adrenal absent, left adrenal intact.

Protocol 3.—Rat 42; white, male. Oct. 20, Active. Weight, 320 gm. Operation, excision of both adrenals, much abdominal fat. Oct. 21. Ate 10 gm. of food. Oct. 22. Active, ate all of food (15 gm.). Oct. 27. Active. Weight, 310 gm. At 11 a.m. injected subcutaneously 0.62 cc. of 1 per cent morphine sulfate solution (= 20 mg. per kg.). 1:30 p.m., marked pilomotor effect. Did not move. 6 p.m., very quiet. Oct. 28. Very quiet, not eating. Oct. 29. Quiet. Ate a little today. Oct. 30. Very dull, not eating. 5 p.m., comatose. 9:30 p.m., found dead.

Autopsy.—Weight 277 gm. Diarrhea. Thyroid large and dark red. Heart and lungs normal. Abdomen: scant fat. Both adrenals absent. No accessories seen. Right testicle congested. Liver and spleen normal. Stomach: one hemorrhage beneath mucosa near fundus.

Protocol 4.—Rat 21; white and brown, male. Sept. 27. Active. Weight 345 gm. Operation, excision of left adrenal, easy operation. Oct. 4. Active, eating well. Weight 333 gm. At 10:48 a.m. injected 0.75 cc. of 2 per cent morphine sulfate intravenously (= 45 mg. per kg.). 10:50 a.m. respirations imperceptible. Board-like rigidity. 11:10 a.m. respirations very shallow. Complete coma. 1 p.m. condition gradually improving. Oct. 5. Alert and active, eating well. Oct. 11. Active, eating well (15 gm. per day). Weight 335 gm. Operation, excision of right adrenal. Easy operation, no hemorrhage. Moderate fat. Oct. 18. Excellent condition, active, good appetite. Weight 321 gm. At 3:50 p.m. injected 0.74 cc. of 1 per cent morphine sulfate intravenously (= 23 mg. per kg.). 3:52 p.m. respirations imperceptible for a minute, and rat is stiff. 3:57 p.m. voluntary movement. Oct. 19. 9 a.m. very quiet. Marked ruffling of fur. Not eating. 6 p.m. very quiet and dopey. Oct. 20. Found dead this morning.

Autopsy.—Well preserved fat. Diarrhea. Thyroid rather large and red. Heart and lungs normal. Abdomen: both adrenals absent. No accessories seen. Stomach: five distinct hemorrhages into mucous membrane, one is 3 x 4 mm.

Protocol 5.—Rat 24; white and gray, male. Sept. 27. Active. Weight 320 gm. Operation: double adrenalectomy. Easy operation. Sept. 29. Eating well, alert. Oct. 2. Active, ate 12 gm. Weight 318 gm. Oct. 3. Ate 10 gm. Oct. 4. Active. Weight 311 gm. Ate 12 gm. At 10:30 a.m. injected 0.28 cc. of 1 per cent morphine sulfate intravenously (= 9 mg. per kg.). Became quiet but did not stop breathing. 5 p.m. quiet and fur ruffled. Oct. 5, very quiet. Eyes closed most of time. Hunched up, fur ruffled. Drinks with eyes closed. Not eating. Oct. 6. Head between front paws most of time. Pilomotor reaction marked. Oct. 7. Condition about the same. Has eaten nothing since

operation. Oct. 8. Seems slightly more active, not eating. Oct. 9. Ate 5 gm. of food, first since injection. Oct. 10. Very drowsy. Stools firm and white. Not eating. Oct. 14. Very weak. Has not eaten 10 gm. since injection. Oct. 15. Found dead this morning.

Autopsy.—Weight 240 gm. Thyroid moderate size. Heart in systole. Lungs normal. Abdomen: adrenals absent. No accessories seen. Left testis converted into a sack filled with dark red grumous material. Right testis atrophied. Stomach: postmortem change.

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ON THE ANTIGENIC PROPERTIES OF HEMOGLOBIN.*

By M. HEIDELBERGER, Ph.D., and K. LANDSTEINER, M.D.

(*From the Hospital and the Laboratories of The Rockefeller Institute for Medical Research.*)

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The antigenic properties of hemoglobin[1] are of interest because there is available in the form of the oxy-compound a crystalline substance which, unlike most other animal proteins, appears to be a chemical individual and can be tested for purity by chemical methods. For these reasons much work has been done on the subject, but the conclusions reached have been conflicting.

Leblanc,[2] Ide,[3] and Demees[4] were the first to report the immunization of animals with hemoglobin. Ide's antiserum was hemolytic in addition, but his pupil Demees, by purifying his hemoglobin more thoroughly, succeeded in obtaining a serum which precipitated hemoglobin solutions and did not cause hemolysis. Thorough purification is important since the blood corpuscles contain substances of stronger antigenic power (globulins, stromata) than hemoglobin (Ide). The precipitins obtained by Demees were not bound by intact red cells, and were produced only by intensive treatment of the animals.

The question of the species-specificity of hemoglobin was investigated by Thomsen[5] with the aid of the anaphylactic reaction. Most of his work was carried on with dissolved corpuscles, though he made a few experiments with the crystalline hemoglobin of two species. Similar experiments were made by Bradley and Sansum.[6] Guinea pigs sensitized with dog hemoglobin reacted strongly (but not

* Seventeenth paper on antigens.

[1] The word hemoglobin, in this communication, is used in the generic sense, including oxyhemoglobin and its immediate derivatives.

[2] Leblanc, A., *La Cellule*, 1901, xviii, 337.

[3] Ide, M., *La Cellule*, 1902, xx, 263.

[4] Demees, O., *La Cellule*, 1907, xxiv, 423.

[5] Thomsen, O., *Z. Immunitätsforsch., Orig.*, 1909, iii, 539. *Cf.* Schittenhelm, A., and Weichardt, W., *Z. Immunitätsforsch., Orig.*, 1912, xiv, 609. Pfeiffer, H., and Mita, S., *Z. Immunitätsforsch., Orig.*, 1910, vi.

[6] Bradley, H. C., and Sansum, W. D., *J. Biol. Chem.*, 1914, xviii, 497.

with acute death) to solutions of dog red cells, less markedly to those of the cat, pig, and turtle, and not at all to the blood of a number of other animals.

As there is no reason to believe that the hematin in the hemoglobin molecule of various species is different, and it is at least certain that there are not many different hematins, the differences in the antigenic properties of the different hemoglobins would appear to be due to the globin, or the protein part of the molecule. The serological behavior of globin was studied by Browning and Wilson;[7] also by Gay and Robertson.[8] The former obtained, in addition, an antihemoglobin serum and found that an immune serum against guinea pig globin fixed the complement strongly with the corresponding antigen, and reacted only weakly with rabbit globin and not at all with ox globin. In a second paper Browning and Robertson report that ox globin antiserum reacts, in addition, with the globins of the goat, guinea pig, and duck, but not with that of the rabbit. They write: "Thus while evidence of species-specificity exists in certain cases, there is also a wide, though not universal, community of antigenic properties shared by the globin of widely separate animal species."

In contrast to the positive results cited above, Ford and Halsey[9] were unable to produce either antibody formation or anaphylaxis by injecting repeatedly recrystallized hemoglobin. Similar negative results with both hemoglobin and globin were reported by Schmidt and Bennett,[10, 11] using hemoglobin which had been carefully purified by various methods. They therefore concluded that hemoglobin is a non-antigenic substance and that the positive findings of previous workers were possibly due to the impurity of the hemoglobin or globin used. Subsequent to their work positive findings were again reported by Fujiwara.[12]

Differences between the various antigens contained in the blood corpuscles have been investigated by Klein, Leers, Levene, Chodat, Steward, and Fujiwara.

The solution of the question of the antigenic or non-antigenic nature of hemoglobin is of considerable general interest, since if it were really impossible to produce antibodies with hemoglobin, doubts might arise as to the antigenic properties of absolutely pure proteins in general. It would seem far more reasonable, however, to view such behavior as pointing to a relationship between the con-

[7] Browning, C. H., and Wilson, G. H., *J. Path. and Bact.*, 1909, xiv, 174; *J. Immunol.*, 1920, v, 417.

[8] Gay, F. P., and Robertson, T. B., *J. Exp. Med.*, 1913, xvii, 535.

[9] Ford, W. W., and Halsey, J. T., *J. Med. Research*, 1904, xi, 403.

[10] Schmidt, C. L. A., and Bennett, C. B., *J. Infect. Dis.*, 1919, xxv, 207. Schmidt, C. L. A., *Univ. Calif. Pub.*, 1916, ii, 157.

[11] Bennett, C. B., and Schmidt, C. L. A., *J. Immunol.*, 1919, iv, 29. *Cf.* Gay, F. P., and Robertson, T. B., *J. Exp. Med.*, 1913, xvii, 535.

[12] Fujiwara, K., *Mitt. med. Ges. Tokyo*, 1920, xxxiv, No. 23.

stitution of individual proteins and their antigenic action. Moreover, the determination of the species-specificity of hemoglobin antibodies, if they really exist, would have importance as throwing light on the general problem of how species-specificity is manifested in the individual proteins of the animal organism. It is from this standpoint that we have taken up the matter, and a preliminary note has been published in which this phase of the problem is discussed more thoroughly.[13] It has developed that antibodies can be obtained with hemoglobin, although less readily than with most other proteins, and that these antibodies are species-specific to a high degree.

After the publication of the preliminary note, and after the completion of the present work, a communication by Higashi[14] was received in which the author's conclusions coincide essentially with our own. On the other hand, negative results were published by Depla.[15]

Recently, Hektoen and Schulhof[16] reported the production of antibodies with blood extracts and hemoglobin solutions, concluding that hemoglobin is a species-specific antigen. Contradictory in a certain measure, however, is their finding that there was no appreciable diminution of precipitable substance after fission of the hemoglobin into hematin and globin and removal of most of the split products. Their conclusion is: "While the precipitinogens in extracts of red corpuscles and in hemoglobin may exist independently of hemoglobin after treatment with acids, they ordinarily are attached closely to the hemoglobin molecule, not being removed or diminished in proportion to the amount of hemoglobin by repeated crystallization or by treatment with aluminium cream, the antigen being apparently either closely adsorbed to the hemoglobin molecule or forming a part of it which can be split off by acids." We shall discuss this conclusion further on in our paper.

Preparation of Oxyhemoglobin and Immunization of the Animals.

In the work reported in this paper horse hemoglobin only was used for immunization.

The preliminary experiments referred to above were carried out with a preparation (A) made as follows, according to the methods of Ide[3] and Demees.[4]

[13] Landsteiner, K., *Kon. Akad. van Wetensch. te Amsterdam*, 1921, xxix, 1029.

[14] Higashi, S., *J. Biochem.* (Japan), 1923, ii, 315. *Cf. J. Tokyo Med. Soc.*, 1921, xxxv, No. 9.

[15] Depla, H., *Compt. rend. Soc. biol.*, 1922, lxxxvii, 383. *Cf.* Chodat, F., *Compt. rend. Soc. biol.*, 1921, lxxxv, 735.

[16] Hektoen, L., and Schulhof, K., *J. Infect. Dis.*, 1922, xxxi, 32.

Defibrinated horse blood was centrifuged, washed five times, and dissolved in a volume of water equal to twice the amount of blood used. Ether was added, and the mixture allowed to stand in the ice box for 1 or 2 days, with occasional stirring. After decantation from any sediment present, the solution was treated with an equal volume of saturated ammonium sulfate solution, and filtered through folded filters to remove precipitated globulin and stromata. Saturated ammonium sulfate was then added until the hemoglobin separated in the cold. The product was filtered off, washed with ammonium sulfate solution, and dialyzed after the addition of a little ether. Finally, 1 per cent of sodium chloride was added.

Preparation B, a solution of oxyhemoglobin, was obtained according to the method recently published by one of us.[17]

The oxyhemoglobin was recrystallized three times as outlined in the method referred to, and washed several times with water saturated with carbon dioxide and oxygen. The crystals were ground to a thin paste with 0.85 per cent sodium chloride solution, diluted further with saline, dissolved with the minimum amount of normal sodium hydroxide solution, and diluted with saline to a total hemoglobin content of 8 to 10 per cent. The solution was first filtered through a Berkefeld V filter, then through a sterile Berkefeld N filter, and was preserved under sterile conditions.

Preparation C, also a solution of oxyhemoglobin, was prepared by following the method used for Preparation A as far as the addition of an equal volume of saturated ammonium sulfate solution.

It was found that if the ether used was repeatedly washed with water, and then dried first over calcium chloride and then over stick potassium or sodium hydroxide, the formation of methemoglobin was avoided. In fact, crystallization of the oxyhemoglobin occurred so rapidly after addition of the ammonium sulfate solution that it was rarely possible to filter the mixture without losing much of the pigment. The filtrate from the globulin and stromata was allowed to stand overnight in the ice box and the solution was then decanted from the heavy deposit of oxyhemoglobin crystals. These were filtered on a Buchner funnel and washed, and were recrystallized once and dissolved according to the methods used for Preparation B, omitting the preliminary filtration through a Berkefeld V filter.

8 to 10 per cent hemoglobin solutions prepared as above were used for injection. Seventeen rabbits in the three groups each received seven to eleven intraperitoneal injections of 8 to 15 cc. at weekly intervals. Only with a small proportion of the animals did we obtain strongly reacting sera, that is, sera which gave a heavy precipi-

[17] Heidelberger, M., *J. Biol. Chem.*, 1922, liii, 31.

tate with 0.01 per cent hemoglobin solution within a few minutes. In the case of Preparation A one out of five rabbits gave a strongly reacting serum and two gave weaker sera; with Preparation B, two out of five rabbits gave strongly reacting sera, while with Preparation C, in spite of the use of seven rabbits, only weak sera were obtained, that is sera which, under the conditions given below, gave readily perceptible, but relatively weak turbidities or slight precipitates.

Thus we have found, as did Demees[4] and Browning and Wilson,[7] that hemoglobin is a rather weak antigen, a fact which may account for the negative results of the experiments of others. Possibly, however, a greater formation of antibodies could be achieved through intravenous injection as was done by Hektoen and Schulhof. It is also not impossible that some of the impurities associated with hemoglobin in the cell may actually facilitate antibody formation by the hemoglobin. By the use of the especially sensitive ring test, as employed by Hektoen and Schulhof[16] and Higashi,[14] other sera would undoubtedly have been found positive.

The Specificity of Antihemoglobin Sera.

The tests were carried out as follows: 2 to 3 capillary drops (1 drop = 0.04 cc.) of the serum were added to 0.2 cc. of 0.01 per cent solutions of the different hemoglobins. In Table I is given the intensity of the turbidity or precipitate after the tubes were shaken and had stood for various periods at room temperature. Solutions of crystalline horse and dog oxyhemoglobin were used in the tests, while in the case of other animals, red cells were laked with water and ether, centrifuged, and the resulting solution was filtered through asbestos or kieselguhr paper (Macherey) and made up to a definite hemoglobin concentration. Serum 1 was obtained with Preparation A, the others, with Preparation B.

After the test mixture had stood overnight in the ice box, some further weakly positive reactions were noted. Precipitin reactions of equal intensity were obtained with methemoglobin, carbon monoxide hemoglobin, and cyanhemoglobin prepared from crystalline horse oxyhemoglobin.

The reactions in Table I show that the serological specificity of the antibodies produced by crystalline hemoglobin is no less developed than in the case of the serum proteins, which have been repeatedly and thoroughly studied from this standpoint (Nuttall[18]). Strong

[18] Nuttall, G. H. F., Blood immunity and blood relationship, Cambridge, 1904.

TABLE I.

Specificity of Antihemoglobin Sera.

	Read after	Human.	Dog.	Horse.	Donkey.	Pig.	Ox.	Sheep.	Goat.	Rabbit.	Guinea pig.	Rat.	Mouse.	Chicken.	Pigeon.
Serum 1	1 hour	0	0	+++	++±	0	0	0	0	0		0	+	0	0
" 2	5 min.	0	0	++±	++	0	0	0	0	0	0	0	0	0	
" 2	20 "	M.Tr.	0	+++	++±	0	0	Tr.	0	0	0	0	0	0	
" 2	1 hour	" "	0	+++	+++	0	M.Tr.	"	M.Tr.	0	0	0	M.Tr.	0	
" 3	20 min.	0	0	++±	++	0	0	0	0	0	0	0	0	0	
" 3	30 "	0	0	++±	++	0	0	0	0	0	0	0	0	0	
" 3	1 hour	0	0	++±	++	0	0	0	0	0	0	0	M.Tr.	0	
" 4	30 min.	0		+±	+		0						" "		
" 4	1 hour	0		++	+±		0						" "		

Tr. indicates trace; M.Tr., minimal trace.

reactions were given with the homologous horse hemoglobin and a somewhat weaker precipitation with the blood pigment of the closely related donkey, while the other red cell solutions gave little or no reaction. This marked precipitation of the hemoglobin of a closely related species parallels the findings with serum proteins, and shows that with hemoglobin as well, the serological or chemical relationship runs parallel to the morphological. In agreement with this conclusion, already stated in the preliminary paper,[13] Higashi[14] has likewise found a definite species-specificity in precipitin, complement fixation, and anaphylactic reactions. An exception was found by him in the case of closely related species. Moreover, anti-chicken and anti-goose hemoglobin sera gave reactions of equal or nearly equal intensity with the hemoglobin of the pigeon and sparrow.

The serological findings are manifestly a proof that the crystallographic differences found by Reichert and Brown[19] in their comprehensive work are principally the result of chemical differences in the hemoglobin, a conclusion which has hitherto not been universally accepted,[20] since conditions of crystallization and impurities present undoubtedly do influence crystalline form.[21]

With respect to the serological differences between hemoglobin and other proteins, our immune sera, like those obtained by Demees and Higashi, had a very weak hemolytic action and either gave no reaction at all with horse serum albumin, or only a very faint trace on long standing. On the other hand, it was possible, by means of the inhibition reaction,[22] to trace a relationship between hemoglobins of different origin. The observed effect was less, however, than that recorded in our preliminary publication.

To each of a number of tubes was added 0.2 cc. of 0.01 per cent horse hemoglobin solution and 0.1 cc. of a solution of red cells of the animal indicated and con-

[19] Reichert, E. T., and Brown, A. P., *Carnegie Inst. Washington, Pub. No. 116*, 1909. *Cf.* Loeb, J., *Science*, 1917, xlv, 191.

[20] *Cf.* Robertson, T. B., Principles of biochemistry, Philadelphia and New York, 1920.

[21] For additional evidence of chemical differences see Landsteiner, K., and Heidelberger, M., *J. Gen. Physiol.*, 1923–24, vi, 131.

[22] (*a*) Camus, M. L., *Compt. rend. Acad.*, 1901, cxxxii, 215. (*b*) Landsteiner, K., and Halban, T., *Münch. med. Woch.*, 1902, xlix, 473. (*c*) Landsteiner, K., *Biochem. Z.*, 1918–19, xciii, 115; 1920, civ, 280.

taining approximately 0.1 per cent of hemoglobin. 0.1 cc. of 1 per cent of sodium chloride solution was added to a control tube and 0.08 cc. of Serum 3 was put in all. The results are shown in Table II.

It is not unlikely that the inhibition observed in these tests depends upon the existence in the various hemoglobins of a common component, such as hematin, in view of the analogous inhibition shown in the case of proteins containing a common radical which had been introduced into the molecule.[22c] However, a solution of hematin itself caused no inhibition, just as it failed to give reactions with precipitin sera.[23]

TABLE II.

Inhibition of Precipitation.

Dog.	Horse.	Rabbit.	Chicken.	Control.
Tr.	0	+ −	+	++

Identity of the Precipitable Substance with Hemoglobin.

Because of the negative results of many authors, the difficulty of producing strongly reactive antibodies, and the observations of Hektoen and Schulhof, it is of great importance to settle the question as to whether the antigen in our experiments is really hemoglobin itself, or some impurity difficult to remove.

The most striking result reported by Hektoen and Schulhof was that the precipitin reaction remained unaltered in solutions in which the hemoglobin had been destroyed with acetic acid and the globin removed. However, hydrochloric acid, when not greatly diluted, destroyed the antigen.

We repeated the experiments with acetic acid and hemoglobin at room temperature in the concentrations given by the authors, removing test portions from time to time. After removal of the globin by neutralization, a series of dilutions was made and compared with dilutions of the original hemoglobin solution. Precipitin tests were made on these solutions, and rough estimations were made of how much of the original antigenic value remained, by comparing

[23] Gay, F. P., quoted by Schmidt and Bennett.[11]

the acid-treated tubes with the hemoglobin dilutions. In this way it was found that after ½, 2, and 22 hours, about 50, 60, and 75 per cent, respectively, of the antigen had been destroyed. Part of the hemoglobin was still spectroscopically demonstrable even after the action of 0.01 N hydrochloric acid instead of 0.1 N acetic acid for 24 hours.

In view of this fact, the following experiment was set up.

A 10.2 per cent solution of twice recrystallized oxyhemoglobin was prepared by dissolving a suspension of the crystals with the aid of the minimum amount of normal sodium hydroxide. 50 cc. of the solution were diluted to 100 cc., and although neutral to litmus, the solution was acidified with 2 cc. of normal acetic acid in order to avoid any question as to its reaction. 50 cc. of the resulting 5 per cent solution were mixed with 50 cc. of 0.05 N hydrochloric acid and allowed to stand at room temperature for 4½ hours. The brown solution was then neutralized with 0.1 N ammonium hydroxide containing 5 per cent of ammonium chloride, allowed to stand overnight, and centrifuged. A determination of hemoglobin by Stadie's method[24] showed 0.2 of the original amount still to be present, while a similar solution, allowed to stand overnight before neutralization, still contained 0.1 of the original amount. The solutions showed the absorption bands of methemoglobin, and reacted with immune serum with approximately the degree of intensity to be expected from the analytical findings.

It thus becomes evident that, under the conditions given, hemoglobin is not completely split into hematin and globin.

The fact that the antigen is destroyed to an appreciable extent in a short time by acetic acid of a lower concentration than 0.05 N is in itself strong support for the belief that hemoglobin is the substance active in the serological tests; for other antigens such as the serum proteins are influenced in their precipitin reactions only by acid of much higher concentration.

To sum up, there are a number of grounds for ascribing the antigenic action to hemoglobin itself. In the first place, removal of the stromata by filtration and of globulins by ammonium sulfate followed by recrystallization, leaves the precipitability of the hemoglobin solution intact, as does also treatment with aluminium cream according to Marshall and Welker,[25] as Hektoen and Schulhof found. In

[24] Stadie, W. C., *J. Biol. Chem.*, 1920, xli, 237.

[25] Marshall, J., and Welker, W. H., *J. Am. Chem. Soc.*, 1913, xxxv, 820.

the second place, the observation that our immune sera reacted to an equal extent with solutions of purified hemoglobin and with extracts of red cells containing all the accompanying impurities points in the same direction. In the third place, the precipitin reaction permits the detection of hemoglobin in as low a concentration as 0.001 per cent, which compares favorably with the strength of common precipitins. It would be difficult to reconcile this fact, and that of the inhibition found at a concentration of 0.05 per cent, with the assumption of an active impurity. Finally, it can be shown that hemoglobin is actually carried down in the precipitate formed in hemoglobin solutions by immune serum. The important observation that the precipitate is red was originally made by Leblanc and Ide.[26] Since it could be objected that the hemoglobin might have been carried down by adsorption, and not have been actually involved in the precipitin reaction, tests were made under conditions which eliminate this possibility.

The following immune sera of rabbits were used.

A. Antihemoglobin Serum 2	20 drops
B. " " 3	20 "
C. Anti-horse serum	10 "
D. Anti-human "	20 "
E. Anti-donkey "	20 "

The tubes were set up as follows:

In each tube 0.2 cc. of 0.1 per cent hemoglobin solution plus

1. 1.8 cc. saline + A.
2. 1.8 " " + B.
3. 1.8 " 1:500 horse serum + C.
4. 1.8 " 1:500 human " + D.
5. 1.8 " 1:500 donkey " + E.

At the end of 1 hour the precipitates were centrifuged off, washed with saline, and again centrifuged. The differences observed were very striking. The sediment in Tubes 1 and 2 was definitely red, while that in the other tubes was pure white in spite of the fact that the antigen-antibody complex was precipitated in a hemoglobin solution of the same concentration as that in the first two tubes. One is forced to the conclusion that the red color of the precipitate in the hemoglobin-antihemoglobin system is actually due to antigen-antibody combination.

The following additional experiment was run.

1 cc. of 8 per cent hemoglobin solution was diluted with water to 30 cc. and treated with 20 cc. of 0.01 N hydrochloric acid. After 2 hours the globin and

[26] See Leblanc,[2] p. 362; Ide,[3] p. 266.

hematin were precipitated by neutralization. The precipitin test showed in comparison with hemoglobin solutions of known strength that about 80 per cent of the antigen in the pale yellow-brown solution had been destroyed. 1 cc. of the solution was then treated with enough antihemoglobin serum to induce maximum precipitation, after which the precipitate was handled as in the preceding experiment. In this case, too, the sediment was definitely red, showing that the antibody was bound to a pigmented antigen and not to a colorless split product.

From the above experiments, therefore, it would seem that in order to prove definitely that the antigen or an active part of it can be split from the hemoglobin, it would be necessary to show conclusively that any active solution obtained by treatment with acid contains no hemoglobin, methemoglobin, or closely related derivative.

CONCLUSIONS.

Sera produced by immunization with crystalline oxyhemoglobin react species-specifically with hemoglobin solutions.

Evidence is presented that in this reaction the hemoglobin itself is the active substance.

Conversion of oxyhemoglobin into methemoglobin, carbon-monoxy hemoglobin, or cyanhemoglobin does not alter the response to the precipitating immune serum.

Not only the hemoglobin of homologous species, but also that of other species causes inhibition in greater or less degree of the precipitin reaction.

THE QUESTION OF SENSITIZATION OF JOINTS WITH NON-HEMOLYTIC STREPTOCOCCI.

By HOMER F. SWIFT, M.D., and RALPH H. BOOTS, M.D.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

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These experiments were planned in an attempt to substantiate the hypothesis that the arthritis of acute rheumatic fever is the result of a preliminary specific sensitization of the joints by non-hemolytic streptococci. This hypothesis has been advanced by both Herry (1) and Faber (2), and accepted by certain authors as the most probable explanation of the peculiar type of inflammation found in the joints of patients with this disease.

Herry claims that the peculiarity of the rheumatic lesions is due to an "*endotoxine rhumatismale*" which, he states, he was able to prepare from streptococci grown on solid media by a process of drying, grinding, and extracting with normal saline, and finally removing the bacterial bodies by centrifugalization. He states that the extract from 2 mg. of streptococci was fatally toxic for rabbits, and that the introduction of a few drops of clear extract into the joints of rabbits sensitized these structures so that intravenous inoculation of the animals with homologous streptococci after 8 to 15 days was constantly followed by arthritis in the sensitized joints.

In view of the difficulty experienced by most workers in obtaining endotoxins from any type of streptococci, the claim of Herry that he could produce a powerful toxic substance from a small amount of non-hemolytic streptococcus culture is most surprising. Faber, indeed, was unable to repeat successfully his experiments; and we have likewise failed to obtain an endotoxin from these cocci. Faber, on the other hand, reported that by injecting rabbits' knees with killed cultures of non-hemolytic streptococci these joints were rendered more liable to involvement following subsequent intravenous inoculation with homologous living microorganisms. He, unfortunately, does not state whether the "sensitized joints" were the only joints involved. Because preliminary injection of rabbits' knees with killed pneumococci or typhoid bacilli did not render these structures sensitive to a subsequent intravenous inoculation of the animals with non-hemolytic streptococci, he claimed that "this preparatory or sensitizing process is, within narrow limits, a strictly specific one." It should be pointed out that in his experiments arthritis did not occur in animals first injected

into the joints with killed pneumococci or typhoid bacilli and subsequently inoculated intravenously with homologous living bacteria. If the pneumococcus-treated animals are eliminated from his protocols, cross-sensitization was obtained in only two out of four rabbits instead of two out of ten.[1] This makes it appear that the so called *sensitization* in his experiments was the production of a local lesion which upon healing left a *locus minoris resistentiæ*, a place favorable for the localization of bacteria having a tendency to produce joint lesions. When pneumococci or typhoid bacilli, having little tendency to produce arthritis, were inoculated intravenously into rabbits with joints previously injected this *locus minoris resistentiæ* was not made evident. This seems to us the most probable explanation of Faber's experiments. It is not sufficient, however, to explain the fact that his rabbits inoculated intravenously three times with "Streptococcus 7" had arthritis more frequently than those inoculated only once or twice. We think it most probable that small areas of inflammation were set up in the joints of rabbits following the first or second inoculation, and that these small lesions produced points of lessened resistance. Then, if another strain of streptococci had been used for the third inoculation, joint lesions would have been expected with the same frequency as if the homologous strain had been introduced.

Another way to determine this point would be to inject the right knees of several rabbits with one strain of streptococci and the left with another, and subsequently to inoculate half of the animals with one of the strains and the remainder with the other. If arthritis occurred only in the knees of animals in which the same strain was used for both intraarticular injection and intravenous inoculation one might conclude that specific joint sensitization had been induced. If, on the other hand, arthritis occurred in both knees or in other joints, the probability that specific sensitization had taken place would be seriously doubted. In this manner we attempted to elucidate the question.

EXPERIMENTAL.

Method.

In all instances the experimental animals were brown rabbits weighing 1,200 to 2,000 gm. The hair over the knees was clipped closely so that the bony prominences could be easily recognized and the joints minutely inspected. In order to have a more accurate record of the degree of swelling of the knees these joints were measured daily as follows: the widest distance between the internal and external condyles of the femora, and the broadest portion of the upper end of the

[1] Faber (2), Table VI.

tibiæ. As these measurements were over bony prominences it was fairly easy to have them comparable from day to day. They were made with special calipers having a vernier scale reading to 0.1 mm. It may be thought that such measurements are too fine for practical purposes and that conclusions based on them are liable to be faulty, but considerable preliminary work satisfied us that repeated measurements on normal animals are constant to within 0.2 to 0.5 mm., and frequently there is practically no variation; hence it seemed justifiable to record the degree of joint injury in this manner. Notes were also made of redness, swelling, heat, and stiffness. The last symptom, when occurring early, was regarded as a measure of pain; when occurring later, as an indication of the amount of permanent injury. For the first 5 or 6 days following the intraarticular injections the animals were inspected daily, then every 2nd day for about 10 days, and subsequently twice a week until they were inoculated intravenously; then daily inspections were resumed.

In the first three experiments, after preliminary measurements of the knees, these joints were injected with killed streptococci in the amounts indicated in the protocols; the suspensions were prepared as follows: The centrifugates of 18 to 24 hour broth cultures of the cocci were suspended in normal saline and heated ½ hour at 56°C., then tested by plating to insure sterility. The suspensions were so diluted that 0.5 cc. contained the desired amount of streptococci. Most of the joints were injected with an amount corresponding to 0.5 cc. of the original broth culture, as it had been determined in previous experiments that this amount of heat-killed, non-hemolytic streptococci could be injected into the knee joints of rabbits without demonstrable permanent damage. The injections were made according to the method described by Faber; the needle was passed in a proximal direction through the middle of the patellar tendon until it was felt to slip into the bursa under the patella; the suspension was then slowly introduced, a small amount withdrawn into the syringe by suction and reinjected (piston test) in order to be sure that the needle was properly placed; the needle was then quickly withdrawn to prevent leakage into the periarticular tissue. When it was certain that the bacteria had been injected into this tissue the animals were discarded. The bacteria for intravenous inoculation were usually grown for 18 hours in 10 per cent rabbit serum broth, centrifugalized, and resuspended in sterile Ringer's solution. Except with the first culture of Strain Z75, the streptococci had been recently passed several times through rabbits' knees in an attempt to increase their virulence.

The streptococci were all isolated from blood cultures of patients with rheumatic fever, were typical *viridans* strains possessing different biochemical and immunological characteristics, and had decreasing degrees of virulence as follows: A49, A135, 38D, and Z75.

At the postmortem examination the degree of joint involvement was noted, and films from all the joints were prepared and examined microscopically. In some instances there seemed to be an increased amount of clear fluid, but films revealed no exudation of cells; hence these joints were considered normal. Four degrees of inflammation were distinguishable.

Degree.	Gross appearance.	Microscopic appearance.
+++	Yellow, turbid, purulent fluid.	Marked increase of exudative cells.
++	Gray, cloudy or hazy fluid.	Distinct to marked increase of exudative cells.
+	Increased clear fluid.	Distinct increase of exudative cells.
±	Normal amount, clear fluid.	" " " " "

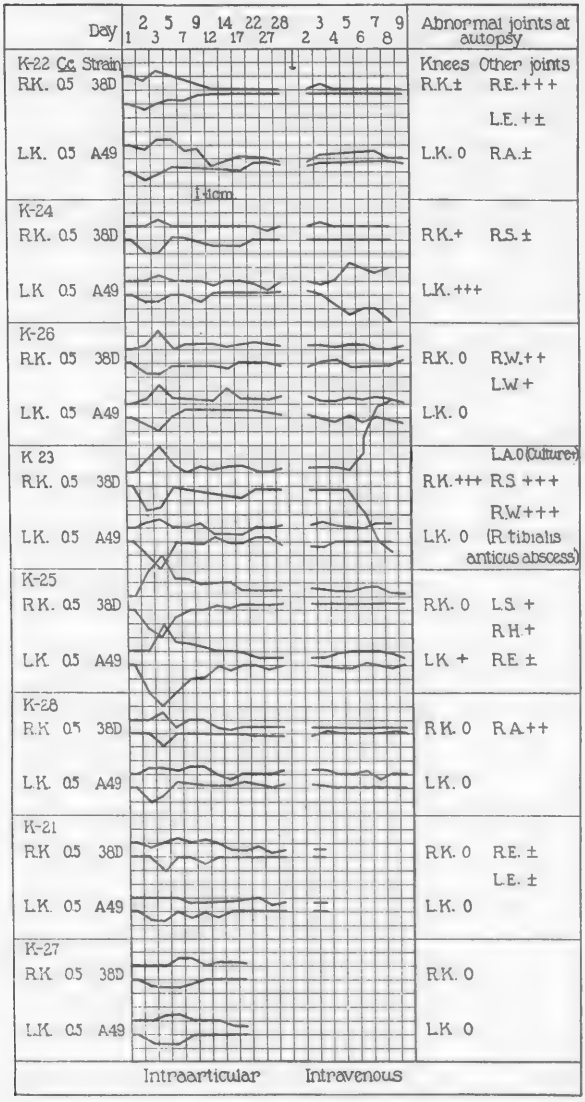
Except in Experiment 4, half of the rabbits received sodium salicylate by stomach tube from the day previous to intravenous inoculation until the time of death. This was done to study the effect of salicylates upon possible joint sensitization, for if bacterial sensitization is the important factor in determining the localization of rheumatic arthritis it would seem probable that salicylates would exert a profound antiarthritic influence. In another communication (3) we have shown that sodium salicylate apparently decreases the intensity of the arthritis in some rabbits inoculated intravenously with non-hemolytic streptococci.

If one considers only the knee involvement among the animals in Experiments 1 and 2, here reported, it would seem as though the salicylates might have exerted an inhibitory influence upon joint sensitization; but consideration of all the inflamed joints makes this less probable.

Explanation of Text-Figures.—The upper line for each knee shows the change in the measurement of that joint through the condyles of the femur; an upward direction of the line indicates increase, a downward direction, decrease. The lower line for each knee shows a change in measurement through the broadest portion of the upper end of the tibia; but here a downward direction of the line indicates an increase, and an upward direction, decrease. The area between these two lines indicates, therefore, the variation in the size of the knees. Broken lines indicate that no measurements were made on that day.

In each chart the blank space between the two periods of observation indicates that this was the last day of the "intraarticular period" and the first of the "intravenous."

R. K. indicates right knee; L. K., left knee; other joints are designated in a similar manner by the initial of the side and joint.



TEXT-FIG. 1. Measurements of knees following intraarticular injection and intravenous inoculation. The arrow indicates the day on which the animals received an intravenous inoculation of the sediment of 9.3 cc. of Streptococcus 38D/4/2.

Experiment 1.—The knee joints of eight rabbits were injected with the sediment of 0.5 cc. of broth culture of killed streptococci as follows: right, with Strain 38D/3/4;[2] left with Strain A49/8/4. The animals were observed for 4 weeks, when it was evident that the inflammation resulting from the intraarticular injection had entirely subsided. 17 days after this first injection, Rabbit K-27 died, evidently from an intestinal infection; both knees appeared normal, and the synovial fluids were free from cellular exudate. This confirms the conclusion derived from clinical observation of the knees, that the injury resulting from the introduction of killed streptococci into them did not persist more than 3 weeks. Salicylate treatment was started with Rabbits K-21, K-23, K-25, and K-28 on the 27th day, and on the next day each of the seven surviving animals was inoculated intravenously with the sediment of 9.3 cc. of serum broth Culture 38D/4/2. The animals were then inspected daily until the 7th, 8th, or 9th day when they were sacrificed and autopsied. Films and cultures were made of all the joint fluids (Text-fig. 1).

Text-fig. 1 shows graphically the reaction of the knees following both the intraarticular injections and the intravenous inoculations and also the degree of inflammation in all abnormal joints at the time of autopsy. Although the animals responded differently to the intraarticular injection, each animal showed a similar amount of swelling in the two knees. It seems evident, therefore, that the two strains of streptococci, when killed, were equally irritating. This is noteworthy when compared with the results obtained from inoculating the two strains intravenously; Strain A49 is much more virulent than Strain 38D. They have different fermentation reactions and are distinct immunologically. One would think, therefore, that if joints were specifically sensitized by having previously reacted to one strain, the intravenous inoculation with that strain would always result in a recurrence of arthritis in that joint, and the opposite knee having previously reacted to the other strain would remain free from a second reaction. This expected result, however, was not obtained in this experiment. Rabbit K-21, dying 2 days after the intravenous inoculation from a Gram-negative bacillus infection, showed no evidence of involvement of either knee, but had slight arthritis in both elbows. Among the six animals surviving the full period of the experiment there were three inflamed

[2] The first figure indicates the strain of streptococcus; the second, the number of passages through rabbits' joints; and the third, the number of subcultures since the last animal passage.

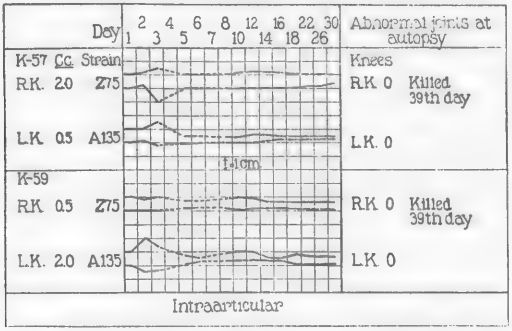
right knees,—the joints injected with the same type of bacteria subsequently used for intravenous inoculation; there were also two inflamed left knees,—the ones previously injected with the other strain; and, in addition, there were twelve other inflamed joints among these animals; also one joint, showing no cellular reaction, from which a pure culture of Streptococcus 38D was grown; this must be considered evidence of bacterial invasion of the ankle in this rabbit (No. K-23). In the seven animals, twenty joints were, therefore, involved, but only three of these were previously injured with the same type of streptococcus that was used for intravenous inoculation. Four knees so injured failed to show any abnormalities post mortem. The knees of one animal (No. K-25) appeared slightly stiff for 3 days after the intravenous injection; this stiffness was thought to be a residuum from the inflammation following the initial joint injury. Both knees of this rabbit showed a marked reaction to the intraarticular injection, but the left, first injured by Streptococcus A49, was the one invaded following the intravenous inoculation with Streptococcus 38D, and three other joints of this animal were also acutely inflamed. Rabbits K-22 and K-23 might be cited as examples of specific joint sensitization if only the knees were considered, but the involvement of three other joints in each animal renders this position untenable.

There seemed to be slight, if any, relationship between the amount of gross reaction following the initial joint injury and the subsequent localization of the living bacteria. By this we do not mean to imply that severe injury to a joint would not be a factor in determining localization of microorganisms in that joint, but rather that the amount of local injury resulting from the intraarticular injection in the rabbits was too slight to have unfavorably influenced the reacting power of the knees. It is probable that only by producing such slight injuries is it possible to study the question of bacterial joint sensitization, for if more severe trauma were inflicted a *locus minoris resistentiæ* would result and doubtless favor the localization of many types of microorganisms circulating in the blood stream.

The cultures from the synovial fluids of all the joints were sterile, with the exception of those from the right knee and the left ankle of Rabbit K-23. The upper half of the right tibialis anticus, and extensor longus digitorum muscles of this animal were markedly inflamed and

yielded a small amount of purulent material on incision. These findings indicate that the animal had comparatively little natural resistance to the infection.

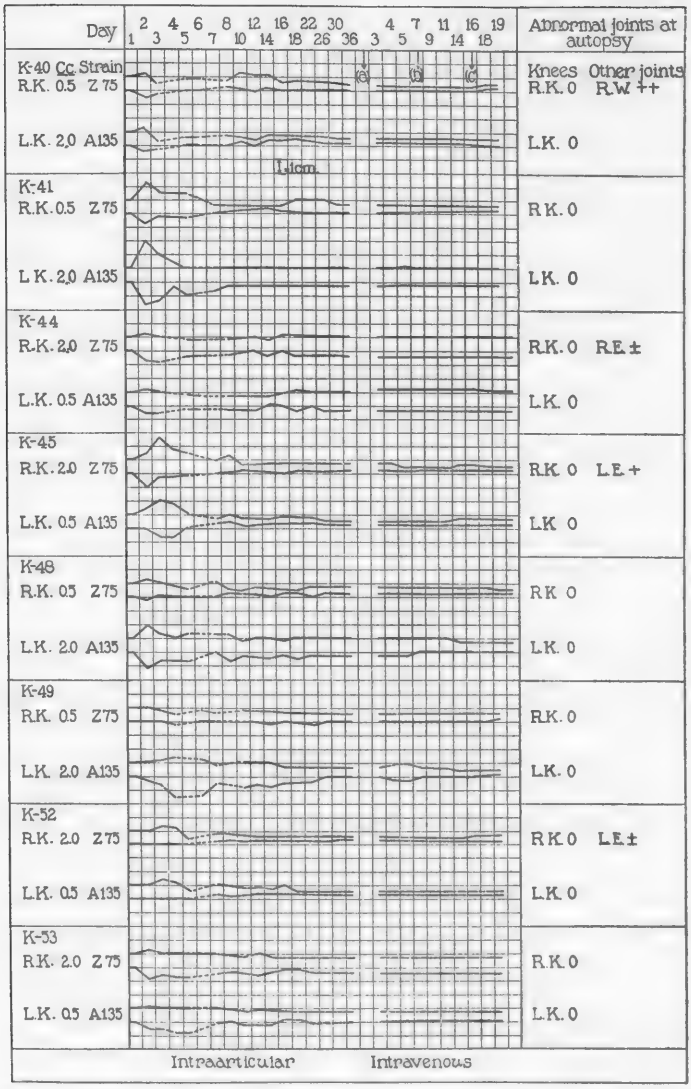
Experiments 2 and 3 serve as controls for one another in that the animals received intraarticular injections of the same streptococci: Strain Z75 in the right knees, and Strain A135 in the left; in half the animals the sediment of 0.5 cc. of broth culture of Streptococcus Z75 and 2 cc. of Streptococcus A135 were used; in the other half the amounts of these two streptococci were reversed. Two animals (Nos. K-57 and K-59) were controls in both experiments; their knee



TEXT-FIG. 2. Measurements of knees of control animals following intraarticular injection with Streptococcus Z75 and Streptococcus A135.

joints were injected in the same manner as in the others and observed for 39 days; then the rabbits were sacrificed and the joints found to be normal (Text-fig. 2). In these two rabbits the knee receiving the larger amount of culture showed the greater reaction, which would be expected; but both knees of many of the animals in the two experiments showed reactions of similar intensity.

Experiment 2.—The knees of eight rabbits were injected, and observed for 36 days, when it was certain that they had recovered from the local injury. On the 35th day half the animals (Nos. K-48, K-49, K-52, and K-53) were given 0.2 gm. of sodium salicylate per kilo of body weight; this treatment was continued until the termination of the experiment. On the 36th day all the rabbits were inoculated intravenously with the sediment of 50 cc. of broth Culture Z75/0/11; 7 days later none showed any evidence of joint involvement, so they were reinoculated with the same amount of Culture Z75/1/2; 8 days later they still failed



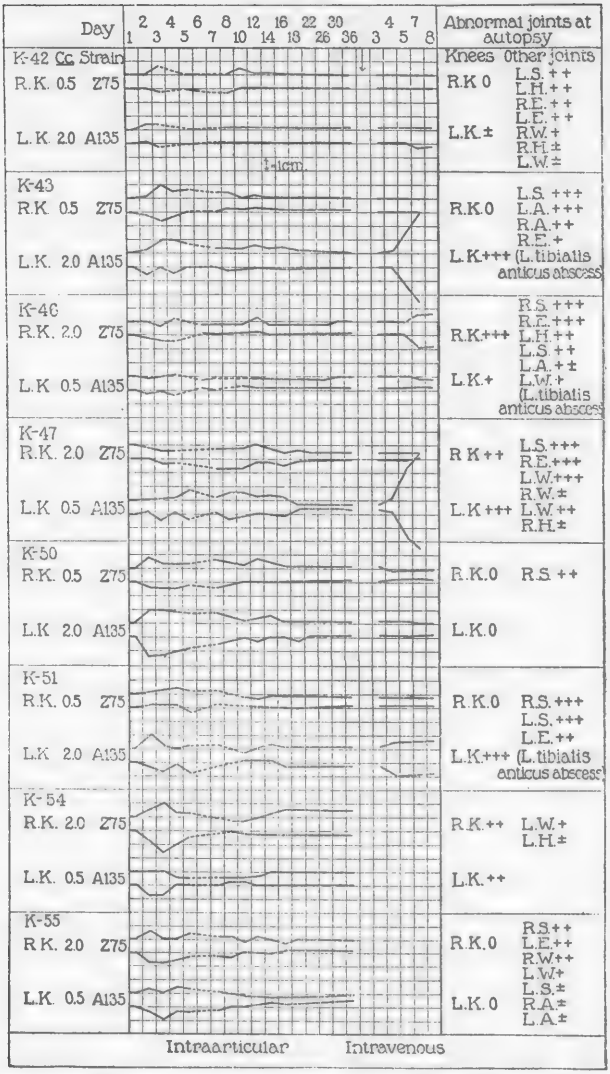
TEXT-FIG. 3. Measurements of knees following intraarticular injection and intravenous inoculation. The arrows indicate the days on which the animals received intravenous inoculations of (*a*) the sediment of 50 cc. of Streptococcus Z75/0/11, (*b*) the sediment of 50 cc. of Streptococcus Z75/1/2, and (*c*) the sediment of 50 cc. of Streptococcus Z75/3/2.

to show any effect from the two inoculations, so were similarly inoculated with 50 cc. of Culture Z75/3/2. 3 days later all appeared well and clinically free from joint lesions; they were sacrificed and examined in the usual manner. The results are shown in Text-fig. 3.

Although most of the injected knees showed some degree of reaction following local injury, none gave any evidence of arthritis following three intravenous inoculations of the animals with large amounts of Streptococcus Z75. Among the eight rabbits, only four joints were found to be definitely diseased. This proved, however, that the inoculum, in the large amounts employed, was sufficient to incite arthritis. The primary injury produced in the right knees of all the animals by injection of this coccus would seem to furnish perfect conditions for the demonstration of specific joint sensitization. The observation that rabbits have more arthritis following repeated intravenous inoculations with non-hemolytic streptococci of low virulence is claimed by Faber to furnish further proof of joint sensitization. Both of the above mentioned conditions, *viz.* intraarticular injection of cocci and repeated intravenous inoculation, were fulfilled in this experiment without any evidence of such sensitization. It will be noted that the salicylated animals had fewer abnormal joints than the non-salicylated; this may be advanced as an argument in favor of eliminating these four animals from consideration, but lack of inflammation in any of the knees of the other four animals, and the results with salicylated animals in other experiments seem of sufficient weight to warrant the inclusion of all in this report.

Experiment 3.—Eight animals were used in this experiment; the knees were injected in the same manner and with the same cultures as those of Experiment 2. On the 35th day half of them (Nos. K-50, K-51, K-54, and K-55) were started on sodium salicylate. On the 36th day all were inoculated intravenously with the sediment of 30 cc. of serum broth Culture A135/2/2. The following day two of the salicylated rabbits (Nos. K-54 and K-55) died; the rest survived for 7 or 8 days, when they were sacrificed and examined as usual. The results are shown in Text-fig. 4.

Streptococcus A135 was much more virulent than Streptococcus Z75 and, therefore, furnished another type of experimental condition. In the entire group there were forty-five abnormal joints, an average of 5.6. The two rabbits (Nos. K-54 and K-55) dying the day after



TEXT-FIG. 4. Measurements of knees following intraarticular injection and intravenous inoculation. The arrow indicates the day on which the animals received an intravenous inoculation of the sediment of 30 cc. of Streptococcus A135/2/2. Rabbits K-54 and K-55 died the following day.

the intravenous inoculation had eleven inflamed joints, an average practically the same as that which prevailed in the surviving group, so it seems justifiable to include them in the general discussion. All the knees injured by intraarticular injection seemed to have recovered completely by the end of the 3rd week; at least, there was no appreciable change in size after that time. There was no constant relation between the reaction of the knees to intraarticular injection and the liability for any given joint to be involved after the intravenous inoculation; for example, both knees of Rabbit K-50 showed the most severe

TABLE I.

Summary of Joints Involved in Experiment 3.

Joint.	Rabbit No.								Total involved.	Per cent involved.
	K-42	K-43	K-46	K-47	K-50	K-51	K-54	K-55		
R. S.	0	0	+	0	+	+	0	+	4	50
L. S.	+	+	+	+	0	+	0	+	6	75
R. E.	+	+	+	+	0	0	0	0	4	50
L. E.	+	0	0	+	0	+	0	+	4	50
R. W.	+	0	0	+	0	0	0	+	3	37
L. W.	+	0	+	+	0	0	+	+	5	62
R. H.	+	0	0	+	0	0	0	0	2	25
L. H.	+	0	+	0	0	0	+	0	3	37
R. A.	0	+	0	0	0	0	0	+	2	25
L. A.	0	+	+	0	0	0	0	+	3	37
R. K.	0	0	+	+	0	0	+	0	3	37
L. K.	+	+	+	+	0	+	+	0	6	75

and persistent reaction, and neither of them was diseased at the time of death. From this animal alone one might conclude that the preliminary articular injury had led to a condition of immunity, but the results in Rabbit K-43 point in the other direction. Both knees of this animal showed intense and fairly persistent swelling following the initial injury, but only the left was involved following the intravenous inoculation; four other joints of this animal were also inflamed, and there was an abscess in the left tibialis anticus muscle. So many foci

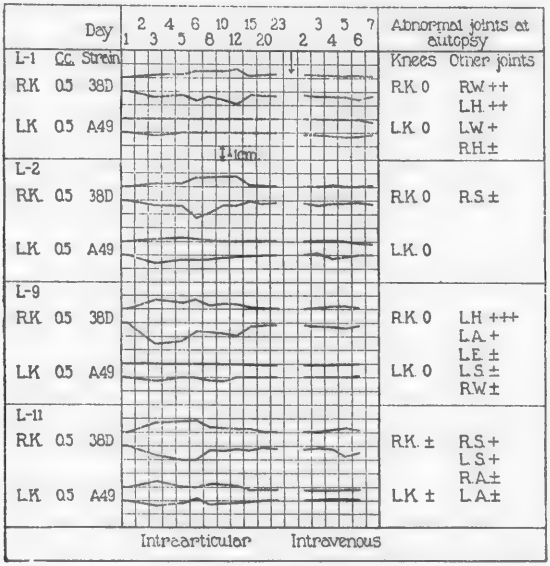
of infection indicate that the animal had much less resistance than did No. K-50.

A summary of the distribution of arthritis among the joints of the different rabbits (Table I) shows that the left knee—the one previously injected with Strain A135—was involved in six animals; the right—previously injected with Strain Z75—was invaded half as frequently. If we consider the percentage of arthritis in the various sets of joints, *i.e.* right shoulder, left shoulder, etc., it is evident that the left shoulder was involved as frequently as the left *sensitized* (?) knee, and that in most instances more joints were affected on one side of the body than on the other. Two of the animals failed, in addition, to show arthritis in either of the previously injected joints; it would, therefore, be illogical to conclude that the localization of inflammation in the left knees of the other six was due to a specific streptococcus joint sensitization. We are inclined to regard the localization in the knees as due to the same factors that determined the localization in other joints—virulence and invasive properties of the streptococci.

Because of the apparent failure to induce joint sensitization with heat-killed streptococci an attempt was made in one experiment to sensitize joints with an extract of streptococci prepared by the method used by Herry. This was considered advisable because of the theory of Zinsser (4, 5) that substances of much smaller molecular size than bacteria may penetrate cells and lead to an allergic state of these cells. It is evident that the experiment as planned by us is not of the same nature as those usually performed to demonstrate sensitization; in the latter the animal is sensitized by introduction of the antigen intravenously or intraperitoneally, possibly subcutaneously, and the local allergic state demonstrated subsequently by introducing the antigen into some particular tissue, as the skin or mucous membrane. This local hypersensitiveness is only a part of a general allergy and cannot be considered the same as the "joint sensitization" described by Herry and Faber.

Experiment 4.—An extract of Streptococcus 38D and one of Streptococcus A49 were prepared as follows: Cultures were seeded on glycerol potato agar to which rabbit blood had been added. After 18 hours the growth was carefully scraped off, taken up in a small amount of saline, and desiccated *in vacuo* over P_2O_5 or KOH. Each extract consisted of two lots; one required 48 hours for

desiccation and the other only 2 hours. The dried bacteria were then pulverized by grinding in an agate mill for 24 hours. The powder was weighed and mixed with sodium chloride solution and water in such proportions that each cubic centimeter contained 4 mg. of dried bacteria and 8 mg. of NaCl. The bacterial suspension was allowed to stand at room temperature with frequent shaking of the container for another 24 hours, when it was centrifugalized at high speed until water-clear. The supernatant solution was pipetted off; 0.5 cc. of Strain 38D was injected into the right knee of each of four rabbits, and 0.5 cc. of Strain A49 into the left. Cultures of the extracts showed that Strain 38D



TEXT-FIG. 5. Measurements of knees following intraarticular injection and intravenous inoculation. The arrow indicates the day on which the animals received an intravenous inoculation of the sediment of 5 cc. of Streptococcus A49/0/10.

was quite heavily contaminated with white non-hemolytic staphylococci, and Strain A49 contained a very few of the same contaminants. No streptococci were recovered from either solution. The local reaction induced by the intraarticular injection might, therefore, have been due to the presence of contaminating staphylococci, as well as to the streptococcus extract. Indeed, the more marked swelling of the right knees when compared with the left suggests that the heavier contamination of Solution 38D was responsible for the severe reaction. It was shown, however, by marked turbidity of the solutions on boiling that they contained a large amount of bacterial protein.

Because of the contamination with staphylococci the solutions were filtered through a Berkefeld filter, and subsequently shown to be sterile. Their toxicity was tested as follows: one rabbit was injected intravenously with 3 cc. of Strain 38D, another with 3 cc. of Strain A49, and a third with 2 cc. of each. None of these animals showed any toxic symptoms. This result would be expected from our general knowledge of the lack of endotoxin in members of the coccus group of bacteria.

23 days after the four rabbits had received intraarticular injections of the two bacterial extracts, each was inoculated intravenously with the sediment of 5 cc. of culture of Streptococcus A49/0/10. They were observed for a week, then sacrificed and examined in the usual way. The results of this experiment are shown in Text-fig. 5.

As already noted, the right knees of all the rabbits reacted quite violently to the intraarticular inoculation. This was probably due to the contamination of the extract. The left knees injected with Streptococcus A49 extract, on the other hand, reacted only mildly. Therefore, at the time of intravenous inoculation the right knee of each animal had been intensely inflamed and possibly sensitized to Streptococcus 38D, and the left knee possibly sensitized to Streptococcus A49. The intravenous inoculation with Streptococcus A49 would be expected to bring out evidence of this sensitization if it existed. The dose was fortunately proper to induce arthritis in all the animals, but only one of them (No. L-11) had inflammation of the knees. Among the four animals there were sixteen inflamed joints, and among these the wrists shoulders, hips, and ankles were affected more frequently than the knees. It is evident, therefore, that the preliminary irritation of the knees of these rabbits did not render them especially liable to localization of the streptococci. The results in general agreed with those of the previous experiments.

DISCUSSION.

A summary of all the results is given in Table II, in which the order of the experiments is arranged according to the percentage of total joints involved in each group. This shows that there was provided a fairly wide range of conditions in respect to virulence and power of the various streptococci to invade joints. In two experiments (Nos. 2 and 4) the injected knees showed less tendency to be involved than did other joints, and the knee previously injected with

killed streptococci or bacterial extract of the same strain that was inoculated intravenously reacted in exactly the same manner as the opposite knee that had been previously injected with another strain of streptococci. In another experiment (No. 1) the knees injected with homologous cocci were involved slightly more often than those injected with heterologous cocci, but the fact that the larger proportion of injected knees failed to be involved following intravenous inoculation of the animals shows that inflammation of some of the knees cannot be attributed to sensitization. In the remaining experiment (No. 3) twice as many knees injected with the homologous strain were involved

TABLE II.

Summary of the Results of All Experiments.

Experiment No.	2	1	4	3	Total.
No. of animals	8	7	4	8	27
Total No. of joints (12 per animal)	96	84	48	96	324
Total No. of joints involved	4 = 4%	20 = 24%	16 = 33%	45 = 47%	85 = 26%
Total homologous "sensitized" knees involved (1 per animal)	0	3 = 43%	1 = 25%	6 = 75%	10 = 37%
Total non-homologous "sensitized" knees involved (1 per animal)	0	2 = 28%	1 = 25%	3 = 37%	6 = 22%
Total No. of joints involved, excluding "homologous sensitized" knees	4 = 4%	17 = 22%	15 = 34%	39 = 44%	75 = 25%

compared with those injected with the heterologous strain; but Streptococcus A135 in the amount inoculated had such high invasive powers that practically half the joints of the rabbits were inflamed. It therefore hardly seems justifiable to attribute the involvement of the injected knees to a specific sensitization.

In considering the total of all the experiments the knees injected with homologous strains showed only a slightly higher percentage of involvement than did other joints, and this can be accounted for largely by the figures in Experiment 3. If a definite hypersensitiveness of the joints had been induced by the experimental conditions a much higher proportion of "specifically sensitized" joints should have been inflamed.

It is impossible to coordinate our results with the conclusions of the workers previously quoted. As already mentioned, our analysis of Faber's experiments leads us to different conclusions from those that he drew; we feel, therefore, that his experiments and ours are more or less in accord. Herry states that he inoculated rabbits intravenously 8 to 15 days following intraarticular injection. Our measurements and observations of the rabbits' knees indicate that the inflammation incited by the intraarticular injection often had not completely subsided by the 12th to 15th day. It is not difficult to understand how rabbits with acutely inflamed knees, when inoculated intravenously, might have an increase of the inflammation in these joints. We cannot agree, however, that these streptococcus extracts are markedly toxic, or that intraarticular injection of them so alters the tissues of the joints that streptococci injected intravenously localize only in the treated joints.

Our experiments have nothing to do with the relation of an arthritis to a general allergic state. It is conceivable that an animal which is hypersensitive to a foreign protein or allergic to a certain bacterium, such as the tubercle bacillus, might have a severe arthritis if the foreign protein, specific bacillus, or bacterial protein were introduced into the joint. The arthritis in this event would be of the same nature as a local inflammation caused by the introduction of the specific antigen into the conjunctival sac or into the subcutaneous tissue. A discussion of a possible relation between this type of reaction and the manifestations of rheumatic fever is beyond the bounds of this article.

CONCLUSIONS.

It was impossible to demonstrate a condition of specific joint sensitization to non-hemolytic streptococci by first injecting the joints of rabbits with small doses of killed non-hemolytic streptococci, or with extracts of these organisms, and subsequently inoculating the rabbits intravenously with homologous living bacteria. Joints so treated were no more liable to involvement than were other untreated joints of the same animals.

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STUDIES ON THE ETIOLOGY OF SNUFFLES IN STOCK RABBITS.

Paranasal Sinusitis a Factor in the Interpretation of Experimental Results.

By JAMES E. McCARTNEY, M.D., and PETER K. OLITSKY, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

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INTRODUCTION.

With the hope of throwing more light on the group of upper respiratory diseases in man, we have sought to study a similar affection in laboratory animals,—rabbit snuffles. In choosing this subject, it was apparent that there would be no lack of material because snuffles is almost ubiquitous among stock rabbits, occurring in endemic form practically all the time, and in epidemic form at periodic intervals. Furthermore, the study concerned an ailment, the incitant of which is still in doubt, although some affirm *Bacillus bronchisepticus*, and others *Bacillus lepisepticus*, as the etiological agent. In view of the prevalence of the disease and the frequency with which the rabbit is employed in experimental work, additional findings in this field should prove of importance to the laboratory worker.

Literature Regarding Etiological Agents.—Bacillus lepisepticus is regarded as the incitant of rabbit snuffles by Beck,[1] Kraus,[2] Roger and Weil,[3] Volk,[4] and others. *Bacillus lepisepticus*, first accurately described in 1887 by Theobald Smith,[5] is now classed with the Pasteurella or hemorrhagic septicemia group of microorganisms. An examination of the articles quoted shows that in no instance has uncomplicated rabbit snuffles, free from septicemia, been induced with this bacillus. Indeed, in the hands of some investigators, as, for example, Volk, this

[1] Beck, M., *Z. Hyg.*, 1893, xv, 363.

[2] Kraus, R., *Z. Hyg.*, 1897, xxiv, 396.

[3] Roger, H., and Weil, E., *Arch. méd. exp. et anat. path.*, 1901, xiii, 459.

[4] Volk, R., *Centr. Bakt., 1te Abt., Orig.*, 1902, xxxi, 177.

[5] Smith, Theobald, *J. Comp. Med. and Surg.*, 1887, viii, 24.

organism, when applied to the uninjured nasal mucous membranes of rabbits, fails to cause snuffles; applied, however, to the scarified mucosa, it induces death, apparently from septicemia, but without clinical signs of snuffles. Similarly, Roger and Weil's experiments demonstrate that this bacillus causes experimental rabbit septicemia which, only at times, is associated with what appears to be a symptomatic rhinitis.

On the other hand, an organism first described by Tartakowsky,[6] and rediscovered by Ferry in 1910[7] as the supposed cause of canine distemper, called by the latter *Bacillus bronchisepticus*, was considered later (1912) by Ferry[8] and by M'Gowan[9] and by Ferry and Hoskins[10] as the incitant of snuffles. In the words of Ferry and Hoskins: "It seems to be evident that the ordinary form of snuffles as encountered in the various rabbitries in this country, characterized by a variable nasal discharge accompanied by sneezing and rubbing the nose, with more or less loss of appetite and weight and a rather subacute or chronic course (which constitutes the large majority of cases), is caused by *B. bronchisepticus;* while the more acute form and the most fatal, in the majority of instances is due to *Bact. lepisepticum.*" These writers do not believe that snuffles is a single entity, and quote Ward[11] who could produce snuffles by injecting rabbits intravenously with either *Bacillus ozænæ*, *Bacillus proteus*, or *Bacillus bronchisepticus*.

A number of other microorganisms has been described by different investigators[12] as causing rabbit snuffles, but the literature fails to confirm their specific relationship.

It appears then from the work of previous writers that the question as to the actual causation of rabbit snuffles is still unanswered. Furthermore, there is no clear conception of the definition of the uncomplicated disease. One group of investigators who maintain that *Bacillus lepisepticus* is the etiological agent regards snuffles as a stage or symptom of septicemia.

The investigations reported in this paper have to do with snuffles as it occurs among laboratory stock rabbits. We shall first describe the disease encountered in such animals so as to present the basis on which our experiments were made.

[6] Tartakowsky, M.-G., *Arch. Sc. biol.*, 1898, vi, 255.

[7] Ferry, N. S., *Am. Vet. Rev.*, 1910, xxxvii, 499.

[8] Ferry, N. S., *Am. Vet. Rev.*, 1912–13, xli, 77; *J. Path. and Bact.*, 1913–14, xviii, 445.

[9] M'Gowan, J. P., *J. Path. and Bact.*, 1911, xv, 372.

[10] Ferry, N. S., and Hoskins, H. P., *J. Lab. and Clin. Med.*, 1919–20, v, 311.

[11] Ward, H. C., *J. Infect. Dis.*, 1916, xix, 153.

[12] Hutyra, F., and Marek, J., Spezielle Pathologie und Therapie der Haustiere, Jena, 4th edition, 1913, ii, 17.

Description of the Disease.

Snuffles as it occurs spontaneously in rabbits kept under laboratory conditions may be divided clinically into different groups: the intermittent and the chronic, which are the commonest types, and the acute, which is infrequent.

Intermittent snuffles is to be noted especially in rabbits which are observed over long periods—many months or even years. A typical attack begins with an acute nasal discharge of a slight amount of thin mucus, which may be transitory and can scarcely be called snuffles. In some cases, the process may go on to a mucopurulent or purulent stage with sneezing, rubbing the nose, soiling and consequent matting of the hair of the paws, loss of appetite and of weight. After a number of days, these symptoms subside and the animal returns to normal apparently. Under conditions which reduce the resistance, to be described later, as also spontaneously, several attacks may occur in the course of a year.

In the chronic form of snuffles, the rabbit has a persistent, white, tenacious, nasal discharge accompanied frequently with crust formation on the nostrils. There is to be noted sneezing when nasal obstruction is present, along with rubbing of the nose and soiling and matting of the hair of the paws which are frequently depilated in small areas. The disease may progress to a destruction of the nasal bones with a deformity simulating in outward appearance luetic destruction of the nose in man. Infrequently there forms a localized abscess about the nasal fossæ. The animal may become adapted to this chronic condition so that there is little, if any, loss of weight, or emaciation.

The acute variety is not common, to judge from our experience with stock rabbits of 800 to 3,000 gm. or more in weight, and a few months to several years of age. Indeed, we believe that this condition can best be seen in breeding establishments where animals are observed from the first weeks of life. When it does occur among laboratory stock it is a manifestation of some underlying disease, such as septicemia. In these cases there is a thin, glairy discharge from the nose, and a rapid weakening of the animal leading to death. From the nasal discharge, blood, and organs, especially the lungs, *Bacillus lepisepticus* can be isolated. Or the acute attack may accompany

pneumonia or other infections and resemble the nasal disorder occurring in a variety of primary conditions such as hay-fever, common colds, influenza, the exanthemata, and seen after exposure to irritants or cold, in man. The failure to distinguish acute, uncomplicated snuffles from the nasal disorder secondary to some underlying disease has led some previous investigators into error when they ascribed to the incitant of snuffles, the etiological agent of the disease responsible for the false "snuffles" they observed.

Sinusitis in Snuffles.

In early experiments we noted the frequency of paranasal sinusitis in apparently normal animals and in those suffering from snuffles. This condition interfered with our procedures to such an extent that an extensive study was begun to determine its nature.

Anatomy of the Rabbit's Nose.—The frequency with which the rabbit suffers from nasal affections is probably due to the complexity of the nasal passages.

The turbinate bones which contain numerous folds are situated on the posterior and lateral walls. The maxilloturbinate, a finely ridged mass of bone, occupies the anterior portion of the lateral wall, while the ethmoturbinate, which consists of much broader folds, is placed posteriorly. Each of these folds or serrations is covered with nasal epithelium so that numerous pockets are present. Moreover, the nasolachrimal ducts discharge into the nasal passages. Besides these structures there are various paranasal sinuses. Congestion of the mucous membrane with resultant swelling may convert the folds or sinuses into closed cavities, thus permitting infectious processes or purulent material to remain localized. The tendency for closed pockets of infection to develop has, we believe, a considerable bearing on the production of the clinical condition known as snuffles and on the interpretation of the results of experimental inoculation.

Occurrence of Sinusitis.—A systematic examination was made of the upper air passages of thirty-four rabbits, among which were animals with snuffles. Included in this number were carefully selected normal rabbits which had been under observation for several months.

The examination included a thorough investigation of all the organs including the brain. The nasal passages were exposed by cutting away the bones of the nose and frontal region of the skull. In order to avoid irritation of the mucous membrane by general anesthetics, such as chloroform or ether, the rabbits were killed by quickly severing the upper cervical spinal cord.

Of the thirty-four rabbits, twenty-three were found to be suffering from intermittent or chronic snuffles. Of these latter, twenty-two had a chronic inflammation of the mucous membrane of the upper nasal passages associated with a purulent paranasal sinusitis.

In the cases of long duration, all the sinuses were filled with thick, white, tenacious, inspissated, odorless pus; while in those of shorter duration only one or two sinuses were so affected. The mucous membrane lining the turbinates and the sinuses was thickened but showed no signs of congestion or acute catarrhal inflammation. In some instances, the thickening of the mucosa resulted in a pocket, the opening of which revealed the firm pus already described. On microscopic examination, the epithelial cells were seen to be necrotic and exfoliated in areas. The subepithelial zone was thickened by an extensive exudation of polymorphonuclear and endothelial leucocytes, or monocytes, but without congestion. Some edema was also present, and in Giemsa-stained preparations, bacteria were noted in the ciliary layer and in the subepithelial zone, mainly phagocytosed. Giemsa's, Gram's, and methylene blue stains of the pus showed numbers of mononuclear cells and somewhat less abundant, polymorphonuclear elements. Many of these cells were disintegrated like those in the pus found, as a rule, in chronic infectious processes. That is, the cell outlines were blurred, the nuclei indistinct, and the cell granules few in number. Numerous broken down cells with granules liberated and scattered were seen. Few bacteria, but these often of different species, were found in phagocytosis as well as outside the cells. The culture findings will be described further on.

Of the thirty-four rabbits, two showed signs of slight snuffles, that is, there was no actual nasal discharge, but merely a dampness about the nostrils, and some sneezing, but none of the other signs of a typical attack, as, for example, soiling or matting of the hair of the paws. Such cases are usually designated in the literature as "beginning snuffles." In both instances was found a chronic inflammatory process of the mucosa and sinuses similar to that met with in chronic snuffles.

Of the original series, nine rabbits were carefully selected as having no ailment whatever, and certainly no obvious signs of nasal affection. These animals were under observation for several months, and were regarded as normal stock. Two of the nine showed the chronic inflammatory process of the mucosa of the nasal passages associated with the purulent sinusitis above described, which is similar in its pathological picture to that exhibited by the animals with typical chronic snuffles.

In another series of 121 rabbits[13] which were carefully selected, individually caged, and kept under a rigid quarantine, five showed moist noses, or what might be considered as slight snuffles. In these five, chronic sinusitis was found. Among the remainder, carefully examined for any possible indication of past or present disease, and determined by this rigid test to be outwardly normal, nine revealed a chonic inflammatory condition of one or more of the nasal sinuses.

Bacteriological Examination.—As Ferry and Hoskins pointed out[10] there is no one organism constantly associated with the condition known as snuffles, although they believe *Bacillus bronchisepticus* causes the chronic, and *Bacillus lepisepticus* the acute disease.

In the present study, the material constituting the nasal discharge was obtained during life by means of a swab, and at autopsy the pus from the sinuses and nasal passages was secured. The nasal discharge and pus were stained and examined microscopically for bacteria and cellular elements. Cultures were made by streaking the pus on rabbit blood agar plates which were incubated for 24 to 48, or more hours, at 37°C.

It may be stated that no one organism was found constantly associated with the condition. The commonest microorganisms isolated were, in the order of their frequency, *Staphylococcus albus*, *Bacillus bronchisepticus*, *Bacillus lepisepticus*, *Micrococcus catarrhalis*, a hemolytic mucoid, Gram-negative bacillus, a diphtheroid, and other unidentified bacteria occasionally. Stained film preparations of the nasal discharge and pus from the upper passages showed, as a rule, an extensive degeneration of cells and a limited phagocytosis of microorganisms, features indicating, as already described, that the exudates were chronic in nature.

When more than one sinus is involved, different bacteria may be present in the various sinuses, as the following protocol illustrates.

Rabbit SA, a stock animal, suffering from snuffles of unknown origin and duration, was killed by pithing. The orbital sinus contained thin, glairy pus which yielded a pure culture of a hemolytic mucoid organism resembling those of the Friedländer group of bacilli. The paranasal sinus was filled with thick,

[13] We are deeply indebted to Doctors Brown and Pearce of The Rockefeller Institute for supplying us with a number of these animals and with other helpful assistance.

white, tenacious, inspissated pus from which a pure culture of *Bacillus bronchisepticus* was obtained. The anterior nares, on the other hand, contained thick, white pus which on culture showed a mixture of both of the microorganisms mentioned.

In this instance, illustrative of other findings, the chronic sinusitis in different regions was associated with two distinct bacteria.

In later observations on more than 50 rabbits the incidence of chronic sinusitis was found to be still greater than in those first studied, both as concerns apparently normal animals and those showing chronic, intermittent, or beginning snuffles.

To summarize, it appears that practically 100 per cent of stock rabbits suffering from chronic, intermittent, or beginning snuffles reveal old, chronic, inflammatory processes of the upper nasal passages, while 9 per cent of apparently normal stock animals, free from any symptoms whatever, exhibit similar chronic purulent conditions of the upper nasal passages. Ordinarily, on casual selection of supposedly normal rabbits, this proportion is still higher and may reach 25 per cent of the total. The bacteria most commonly met with are *Staphylococcus albus*, *Bacillus bronchisepticus*, and *Bacillus lepisepticus*. In other words, in about one-tenth of a carefully selected group of normal stock rabbits and in as many as one-fourth of a casually selected group, chronic infectious processes involving one or more of these microorganisms can be found in the nasal passages, *before any experimental procedure is begun.*

Transmission Experiments.

The facts already presented show the difficulties of interpretation of experimental procedures to induce snuffles. It is desirable, before stating our attempts at transmission, to direct attention to certain other factors which may favor the spontaneous onset of the disease, or symptoms simulating the affection. Dust or mould in samples of defective hay, employed for feeding purposes, may give rise to acute irritation of the nasal passages associated with a certain amount of mucoid discharge and also with conjunctivitis. This condition appears to be due purely to mechanical irritation and not to infection. The disease itself may be induced in apparently normal animals by lowering the resistance, as by subjecting them to low temperatures. An analogy can be found

in the experiments of Mudd and his coworkers [14] in which it is demonstrated that chilling the body surface of man may induce "colds." Another factor in reducing the resistance of rabbits is the injection of killed vaccines.[15] In view of the presence of chronic disease in the nasal passages of rabbits supposedly normal, it is presumed that lowering the resistance of the animals causes an extension or increase of the previously existing inflammatory process, thus converting what may be termed a potential snuffles into an active and intermittent type of the disease.

The importance ascribed to *Bacillus bronchisepticus* and *Bacillus lepisepticus* as the etiological agents of rabbit snuffles suggested to us an attempt to induce the disease *de novo* with these cultures, with due regard for the factors which might interfere with the experimental results.

Experiments with Bacillus lepisepticus Cultures.—A pure culture of *Bacillus lepisepticus* [16] was employed in the following experiment. It was originally obtained from snuffles secretion and its virulence had been enhanced by intrapleural passage through twelve consecutive rabbits.

Thirteen young rabbits (about 800 to 1,000 gm. in weight) were used. These were selected because such are said to be especially susceptible to this microorganism[17] and also to exclude, if possible, previous attacks of snuffles. The material inoculated consisted of a heavy suspension (about 5,000 million per cc.) of an 18 hour growth on blood agar seeded directly from the pleural exudate of the twelfth rabbit. 0.5 cc. of the bacterial suspension was forcibly injected into each nostril by means of a 1 cc. syringe.

Only three of the rabbits showed symptoms of snuffles which appeared on the 6th, 13th, and 13th day, respectively, after inoculation. On these days the rabbits were killed. The autopsy of the first animal revealed thick, white, inspissated pus in the left nasal sinus, and the mucous membrane of the anterior nares

[14] Mudd, S., Grant, S. B., and Goldman, A., *Ann. Otol., Rhinol. and Laryngol.*, 1921, xxx, 1.

[15] For example, of two rabbits injected intravenously with killed cultures of *Bacillus bronchisepticus* and of four injected subcutaneously with vaccines of *Bacillus lepisepticus*, all showed typical snuffles after two to six inoculations at a time when the serum contained agglutinins against these microorganisms in titers of 1:150 to 1:500.

[16] This culture was obtained through the kindness of Doctor L. T. Webster.

[17] De Kruif, P. H., *Proc. Soc. Exp. Biol. and Med.*, 1921–22, xix, 34.

was slightly injected. In stained film preparations of the pus, mononuclear cells predominated over the polymorphonuclears. Most of the cells were degenerated: they were poorly and irregularly stained, the nuclei were indistinct and the granules few in number. There were a few small bacilli present, mostly in various stages of phagocytosis. Cultures yielded hemolytic, mucoid, Gram-negative bacilli in pure state but no *Bacillus lepisepticus*. In the second rabbit there was marked coccidiosis of the liver. A small pocket of inspissated pus was noted in the right nasoturbinal region, but no congestion of the mucosa. Cultures of the pus showed *Bacillus lepisepticus*. The third animal had only a slight mucopurulent discharge from the nose without involvement of the sinuses.

Ten of the animals of this series remained free from snuffles from 8 to 10 days after inoculation. They were all killed at the end of the period of observation to determine the presence of sinusitis. Six were normal, but in the other 4, chronic inflammatory conditions were found in the mucous membranes of the upper nasal passages, together with inspissated pus in the orbital and nasal sinuses of one, and in the nasal sinus alone in the remaining three. From the pus of one of these three, no growth was obtained; from that of the second, both *Bacillus bronchisepticus* and *Staphylococcus albus* were isolated, and from that of the third, a hemolytic, mucoid, Gram-negative bacillus was cultured. The rabbit in which both orbital and nasal sinuses were affected showed in the pus of the orbital sinus *Bacillus bronchisepticus*, and in that of the nasal sinus *Bacillus lepisepticus*.

From the foregoing experiments, it will be noted that of thirteen animals inoculated intranasally with a heavy dose of a culture of *Bacillus lepisepticus*, highly pathogenic for rabbits, only three exhibited snuffles. In two of these three a sinusitis was found, the pathological picture of which revealed an inflammatory condition of long standing, probably antedating the inoculations. From the pus of one of the sinuses, *Bacillus lepisepticus* was recovered, but from that of the other, a different bacillus was isolated. The third rabbit showed no chronic inflammation of the upper nasal passages but a considerable time, 13 days, had elapsed between the injection of *Bacillus lepisepticus* and the onset of symptoms. Ten rabbits remained free from snuffles, but in four of these chronic sinusitis was found, associated with various bacteria. An animal showing two distinct sinus infections had different organisms in the sinuses.

We conclude therefrom that *Bacillus lepisepticus* cannot, by itself, be considered as the incitant of snuffles, for, if a group of supposedly normal stock rabbits be observed over the same length of time em-

ployed in our experiments, findings like those in an inoculated group can be obtained even without manipulation, as we have demonstrated.

We now attempted transmission experiments with *Bacillus bronchisepticus*.

Experiments with Bacillus bronchisepticus Cultures.—Our experience with this microorganism closely resembled those with *Bacillus lepisepticus*.

Of six young rabbits inoculated intranasally, in the manner already described, with a culture of *Bacillus bronchisepticus* in its second generation or first subplant from the growth obtained from snuffles material, only one animal exhibited the disease. This took the form of a slight mucopurulent discharge from the nose, which appeared 10 days after inoculation and lasted for 1 day, after which the rabbit returned to normal. The animals were observed over a period of 5 weeks.

As with *Bacillus lepisepticus*, these results indicate that *Bacillus bronchisepticus* cannot be considered as having a specific relationship to the initiation of snuffles in supposedly normal stock rabbits. It is possible, in view of the evidence already presented, that the induction of transitory snuffles in one instance is apparently due to an acute exacerbation of a chronic inflammatory process in the nasal passages, present prior to the experimental procedure.

Attempts to induce snuffles by the inoculation of these supposed etiological agents having failed, experiments were now made in which the nasal discharge itself, or suspensions of the nasal mucosa from typical cases of the disease in stock rabbits were employed. It was thought that perhaps some non-cultivable agent in these secretions, or the conjoint action of *Bacillus bronchisepticus*, *Bacillus lepisepticus*, and *Staphylococcus albus* which were present in them, could operate to induce the disease.

Experiments with Unfiltered Nasal Secretions.—Two series of experiments were made.

In one series, the nasal secretions from recent and old cases of snuffles were collected by means of a thin cotton nasal swab and then this material was introduced into the nasal cavities of young, supposedly normal stock rabbits.

In another series, the snuffles animal was killed by pithing, or a blow on the head, and the entire length of the nasal mucous membrane was dissected out. This was then ground with sand and 10 cc. of saline solution, and 0.5 cc. of the supernatant fluid injected into each nostril by means of a syringe.

Altogether, material was employed from twelve rabbits with early snuffles; that is, animals killed after the onset of the first definite symptoms of the disease. It was inoculated into twenty-two young rabbits.

Of the twenty-two animals, seven showed clinical snuffles. Of these, six were killed and all showed a marked chronic inflammatory process in the nasal passages with suppuration in one or more sinuses. Table I summarizes the results in the seven positive cases.

In this experiment, as in the preceding ones, no definite evidence of the transmissibility of the disease by means of the nasal secretions from snuffles could be obtained. For in six of the seven positive cases, were

TABLE I.

Rabbit No.	Time after inoculation snuffles appeared.	Sinus infection.		Cultures.
		Right.	Left.	
	days			
1*	10	0	0	0
2	3	+	0	*Bacillus bronchisepticus*, *Bacillus lepisepticus*, and *Streptococcus viridans*.
3	1	+	+	*Bacillus bronchisepticus*.
4	1	0	+	" *lepisepticus* and coliform organism.
5	3	+	+	*Staphylococcus albus*; few *Bacillus bronchisepticus*.
6	4	+	+	*Bacillus bronchisepticus*; few *Staphylococcus albus*.
7	10	+	+	" *bronchisepticus*, *Bacillus lepisepticus*, and *Staphylococcus albus*.

* Rabbit 1 showed snuffles 10 days after inoculation. The symptoms consisted of a scanty mucopurulent discharge which disappeared in 3 days. The animal was not killed.

found old, chronic affections in the nasal passages. From the pathological findings of thickened mucous membrane and inspissated pus in the sinuses, containing cells in varying stages of degeneration with relatively few bacteria, mostly phagocytosed, we infer that this nasal condition antedated the inoculation. Furthermore, the irregularity in the period of incubation and the inconstant presence of any one or any single group of microorganisms, together with the preexisting chronic disease of the nasal passages in supposedly normal controls, all indicate that the inoculations were not specifically related to the appearance of snuffles. The evidence supports our earlier observations

that the inoculations incite a latent condition to become an active disease.

Although the unfiltered material failed to give conclusive results, we now used filtered suspensions of the nasal mucous membranes from snuffles animals and inoculated them both intranasally and intracerebrally in young stock rabbits.

Two stock rabbits with typical snuffles were killed by pithing, and the nasal and sinus mucous membranes were dissected out. The resulting material was ground with sand and 10 cc. of saline solution, centrifuged lightly to remove large particles, and the supernatant fluid was passed through a Berkefeld V candle. The clear filtrate showed no growth on the usual media.

The filtrate, 0.5 cc. into each nostril, was inoculated into each of three young rabbits, but these failed to show any symptoms.

The experiment was repeated three times, employing different sources for the snuffles material, but in all cases the results were negative or indecisive.

Five different samples of filtrates were inoculated intracerebrally in doses of 0.25 to 0.35 cc. into eight young rabbits. In no instance did there develop clinical evidence of snuffles, nor any untoward symptoms.

Thus both the unfiltered and the filtered suspensions of snuffles material, the unfiltered inoculated intranasally, the filtered, intranasally and intracerebrally, failed to induce the disease in supposedly normal stock rabbits.

DISCUSSION AND SUMMARY.

In our experience, covering a large number of rabbits, we have found that the condition known as snuffles falls into different types, the acute and fatal, symptomatic of some underlying infection such as septicemia or pneumonia; and the intermittent, and the chronic. The intermittent and the chronic types considered in this paper are those most commonly present in laboratory stocks.

Our observations point to a widespread prevalence of the disease among rabbits kept under laboratory conditions. This statement might be questioned had the stock we examined been derived from a single source. But the animals were procured from dealers who obtain rabbits in New York, Pennsylvania, Ohio, and as far west as Michigan. The same dealers supply the stock of most of the laboratories in the East. Hence we believe that this disease is found generally prevalent in this part of the country.

We have demonstrated that practically all rabbits with intermittent or chronic snuffles reveal old chronic inflammatory processes of the upper nasal passages associated with thick inspissated pus in one or more sinuses. Moreover, these conditions are also present in about one-tenth of carefully selected, supposedly normal stock rabbits and in one-fourth of a casually selected group, free, during long periods of observation, from any of the symptoms of active snuffles. The peculiar anatomy of the animal's nose which predisposes to the ready formation of enclosed pockets of purulent material may be the cause for the chronicity of upper nasal affections. When the animal's resistance is lowered, the long standing, inflammatory process can flare up into an acute exacerbation, and then show itself as typical snuffles. Various means can effect this: chilling the body, the intravenous injection of foreign proteins such as killed vaccines, or the intranasal inoculation of microorganisms of divers species. Bacteriological examination of the nasal secretions or sinus pus from animals with snuffles and those apparently free of the disease shows the presence in both cases of various microorganisms—*Staphylococcus albus*, *Bacillus bronchisepticus*, *Bacillus lepisepticus*, and others, in order of frequency. Different bacteria may be found in different sinuses in the same animal.

A lack of recognition of these factors has led, we believe, to erroneous conclusions with regard to the inciting agent of the disease. *Bacillus lepisepticus* and *Bacillus bronchisepticus* have been declared the incitants of snuffles. Our experiments, in which an attempt was made to induce the disease *de novo* with these microorganisms, failed. In all cases (with a single exception) in which snuffles followed, there was evidence of an infection which, judging from the condition of the nasal passages and from the cells in the exudates or secretions, had existed before the inoculations were made. Furthermore, the microorganisms recovered from the nasal passages had as a rule no relationship to those in the material inoculated. We attempted also to produce snuffles by inoculating intranasally the unfiltered and filtered suspensions of the ground nasal mucous membranes from typical cases of the disease occurring in stock rabbits. These attempts also failed.

It appears, therefore, that intermittent and chronic snuffles, as it attacks rabbits kept under laboratory conditions, is, as a rule, a sign of an underlying condition—an exacerbation of a chronic inflammatory process in the upper nasal passages, associated with a purulent paranasal sinusitis. The microorganisms recovered are to be looked upon as tending to maintain such conditions but we have still been unable to reproduce typical snuffles with them, employing supposedly normal stock rabbits for the purpose. One may presume that some agent, as yet undetermined, diminishes the resistance of the nasal mucosa, allowing different bacteria to invade and multiply there, thus causing disturbance. In this respect perhaps an analogous condition exists to that which prevails in epidemic influenza and common colds in man.

It is obvious that further work along these lines cannot be properly carried out with rabbits whose antecedent history is unknown. The problem of the incitant of snuffles can best be studied in a breeding stock which is well controlled, one affording an opportunity to observe the animals from an early period of life.

CONCLUSIONS.

A study of snuffles in stock rabbits reveals that this disease is associated with a chronic inflammatory condition of the upper nasal passages together with purulent paranasal sinusitis. The outward signs of snuffles are, as a rule, the expression of an exacerbation of preexisting inflammation in the upper nasal mucosa. While it is conceivable that in early or incipient cases the disease may be uncomplicated and independent of chronic inflammatory processes, we have not been able to observe such cases. Supposedly normal stock rabbits, even those carefully selected and quarantined, exhibit the chronic inflammation just referred to. Such animals cannot therefore be employed for the solution of the problem of the actual incitant, which is, as yet, undetermined.

IMMUNITY STUDIES OF ROCKY MOUNTAIN SPOTTED FEVER.

II. Prophylactic Inoculation in Animals.

By HIDEYO NOGUCHI, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

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Of the many urgent and interesting problems regarding Rocky Mountain spotted fever, that of personal prophylaxis seems to be the most important. The experiments reported here were carried out in an attempt to devise a practical method by which a non-immune population might be rendered immune to the bites of infected ticks.

Ricketts and Gomez[1] early recognized the possibility of developing a satisfactory method of preventive inoculation, and carried out a few experiments in this connection but no adequate quantitative experimental analysis of the factors concerned in the production of artificial immunity. Since we have no pure culture of the etiological agent and cannot recognize it microscopically, we must rely solely upon animal inoculations for quantitative as well as qualitative estimation of the presence of the spotted fever organism. Since the number of causative organisms must bear a definite relationship to the amount of antibodies or the degree of immunity which is produced, a knowledge of the number and virulence of the organisms used is a prerequisite to an understanding of the phenomena of immunization. For example, 1 cc. of the citrated blood of guinea pigs with all the symptoms of spotted fever may contain only 1 or as many as 1,000 minimum lethal doses, but this fact can be ascertained only by careful titration of the material by animal inoculations, and unless the number of minimum lethal doses is determined in each instance, the immunization experiments carried out become meaningless or even misleading. Moreover, since the virus dies out in citrated or defibrinated blood

[1] Ricketts, H. T., and Gomez, L., Studies on immunity in Rocky Mountain spotted fever, *J. Infect. Dis.*, 1908, v, 221.

or serum, even at 4°C., it must be titrated simultaneously with the vaccination or subsequent immunity tests in which it is being used.

The selection of experimental animals is another important factor, and since our aim is to develop methods of protection for the human being, we must choose an animal in which the morbidity and mortality with respect to spotted fever infection approach as closely as possible those of man. The guinea pig excels other animals in this respect. The rabbit is far less susceptible than the guinea pig, and monkeys (*Macacus rhesus*) are extremely susceptible, those of a series of eighteen having shown 100 per cent morbidity and mortality. The age and size of the animal are very important. Full grown males, weighing 500 to 600 gm., are to be recommended; first, because of their comparatively greater resistance to banal laboratory infections and their undiminished and remarkably constant susceptibility to the spotted fever infection, and second, because the characteristic genital lesions are best observed in male animals. The consideration of such factors as just outlined is absolutely essential for formulating a practicable method of prophylactic vaccination.

The ordinary immunological procedures, known to be effective in other diseases, have been subjected to experimental determination of their possible application in the case of spotted fever: (1) the effect of injection of non-infective quantities of live spotted fever virus; (2) the effect of injection of killed virus; (3) the effect of injection of dead virus; (4) the effect of injection of freshly prepared neutral or superneutral mixtures of the live virus with immune serum; (5) the effect of heating and of age upon the immunizing properties of neutral mixtures of the virus with immune serum.

The two strains of spotted fever virus used in the present series of experiments were obtained through the kind cooperation of Dr. R. R. Parker, and we continue to use his designation of them as "Cooper" and "Barlow." The guinea pigs were almost always adult males weighing 500 to 600 gm. The virus was used in the form of citrated blood (equal parts of blood and citrate solution), usually drawn from the heart of the infected guinea pig on the 4th to 6th day of fever, preferably when the scrotal lesions became noticeable. Blood drawn on the 2nd or 3rd day of fever contains, in my experience, far less of the virus. Every specimen of blood was tested for bacterial contami-

nation on ordinary broth and slant agar and was titrated simultaneously with each experiment to determine the number of killing doses which it contained. All the injections of virus, or mixtures containing virus, were made intraperitoneally. The temperatures of the animals were taken every morning during the period of observation; that is, until death or recovery, or, in case no infection developed, for at least 2 weeks. The immune serums used in this work were chiefly from

TABLE 1.

Effect of Non-Infectious Quantities of Virus.

Date of inoculation.	Material.	Amount.	Result.	Immunity test.
1922		*cc.*		
May 10.	Citrated guinea pig blood, Cooper strain (1 M.L.D. = 0.01 cc.).	0.001	No reaction.	June 13, 1922 (33 days), Cooper strain 1 cc. (1,000 M.L.D.); typical infection but recovered.
		0.0001	" "	Similarly tested; typical infection; death in 11 days.
		0.00001	" "	Similarly tested; typical infection; death in 11 days.
Jan. 25.	Citrated guinea pig blood, Barlow strain (1 M.L.D. = 1 cc.).	0.1	" "	Feb. 17, 1922 (23 days), Barlow virus 0.5 cc. (5 M.L.D.); typical infection; death in 10 days.
		0.01	" "	Similarly tested; typical infection; death in 19 days.

rabbits which had passed through a typical but non-fatal infection and had been bled 2 to 3 weeks after the temperature returned to normal.[2] Scrotal lesions were frequently observed in the infected rabbits. In some instances the immune serums came from rabbits which had received a second dose of virus (4 cc. citrated blood of the guinea pig) during convalescence; these serums are designated as "reinforced," and their titers are somewhat higher than those of the convalescent serums. All the immune serums were kept in the refrigerator at 4°C. except at time of use.

[2] Noguchi, H., Immunity studies of Rocky Mountain spotted fever. I. Usefulness of immune serum in suppressing an impending infection, *J. Exp. Med.*, 1923, xxxvii, 383.

TABLE II.

Effect of Injection of Killed Virus.

Date of inoculation, December 8, 1922.

Material.	Killed by.	Amount.	Result of inoculation.	Result of immunity test, 28 days later, against Barlow virus 0.1 cc. (1 M. L. D.).
		cc.		
Citrated guinea pig blood (Barlow + Cooper strain),* 100 M.L.D. in 1 cc.	Chloroform.	1	No reaction.	Typical febrile reaction; recovery.
		0.3	" "	Severe infection with typical scrotal lesions; recovery.
Same.	Ether.	1	" "	Severe infection with typical lesions; recovery.
		0.3	" "	Typical infection; died in 9 days.
		0.1	" "	Severe infection; necrotic skin and ear lesions; recovery.
"	Xylene.	1	Slight fever.	Severe infecton with necrotic lesions of scrotum and ear lobes; recovery.
		0.3	No reaction.	Typical severe infection; recovery.
		0.1	Slight fever.	Severe infection; died in 19 days.
"	Heating to 56°C. for 10 minutes.	0.75	No reaction.	Typical severe infection; recovery.
		0.3	" "	Typical infection; died in 14 days.
		0.1	Irregular fever (secondary infection?).	Typical infection.
Same virus, 1 cc. + immune serum, 1 cc.		1	No reaction.	No infection.
Same virus, 1 cc. + immune serum, 1 cc.		0.3	" "	Slight fever; no symptoms of spotted fever.
Same virus, 1 cc. + immune serum, 1 cc.		0.1	" "	Mild fever; no lesions; recovery.

Titration of virus used for testing immunity. January 5, 1923.

Barlow virus (guinea pig citrated blood).	Result of inoculation.
cc.	
1	Typical infection; killed for virus in 5 days.
0.1	" " died in 14 days.
0.01	Suspicious fever; recovery.
0.001	" " "

* Kept in refrigerator for 20 hours.

Effect of Non-Infective Quantities of Live Spotted Fever Virus.

That guinea pigs which survive a non-fatal but typical spotted fever infection resist subsequent infection has long been known, but whether or not animals receiving too small a quantity of the virus to become infected would acquire increased resistance remained to be determined. Ricketts and Gomez published some observations on this point,[1] stating that, although they had made no systematic attempt to determine the vaccinating properties of minute doses of virus, they had reason to believe that prophylactic vaccination could not be based on this principle. The present experiment shows (Table I) that no appreciable immunity develops under these conditions. Three guinea pigs received non-infective doses of one strain and two of another, and all succumbed to subsequent inoculation 33 and 23 days later, respectively.

Effect of Injection of Virus Killed Either by Chemicals or by Heating.

Chloroform, ether, and xylene were allowed to act upon the virus by shaking with it for 5 minutes. After the mixtures had been in the refrigerator (4°C.) for 20 hours, the clear portion in each case was used for inoculation. Guinea pigs were given the sterilized virus in doses of 1, 0.3, and 0.1 cc., and the animals were tested for immunity 28 days later by inoculation of a single minimum lethal dose of Barlow strain (0.1 cc.). There was no evidence of any increased resistance to the infection.

Another portion of the same virus was heated to 56°C. for 10 minutes in a water bath. Three guinea pigs were then inoculated with 0.75, 0.3, and 0.1 cc., respectively. No infection followed, and 28 days later each animal was given one minimum lethal dose of the virus (Barlow strain, 0.1 cc.). All had severe and typical infections, one dying in 14 days (Table II).

The failure of the animals to derive any immunity from the introduction of the killed virus was apparently due to modification of the immunizing substances by the chemical or physical agents, since the same quantities of virus (100, 30, and 10 M.L.D.) when mixed with immune rabbit serum, proved ample, as will be shown later, to render animals immune to the inoculation of the same test material.

Effect of Injection of Dead Virus.

Although the foregoing experiment had shown that the virus could not be killed without impairment of its immunizing properties, the question remained whether it might not retain its power to confer immunity if allowed to die out under such unfavorable conditions as, for example, low temperature for a long period. Hence several animals, which had been inoculated with specimens of virus kept in

TABLE III.

Effect of Injection of Dead Virus.

Strain.	Kept at 4°C. for a period of.	Amount.	Result of inoculation.	Result of immunity test, 40 days after inoculation, against Barlow virus 1 cc. (100 M. L. D.).
	days	*cc.*		
Cooper Guinea pig 11	69	2	No reaction; died of secondary infection in 9 days.	
Same	69	4 (of 1:6 solution in citrate).	No reaction.	Typical infection; died in 16 days.
Cooper Guinea pig 19	57	2	" "	Typical infection; died in 12 days.
Cooper Guinea pig 30	31	2	Typical infection; killed for material.	
Cooper Guinea pig 31	28	2	No reaction.	Typical infection; finally recovered.
Cooper Guinea pig 47	21	1	" "	Typical infection; died in 15 days.
Barlow Guinea pig 24	38	2	" "	Typical infection; died in 12 days.

the refrigerator (4°C.) for periods of 28 to 69 days and had had no infection, were tested 40 days later for immunity by the injection of 100 M.L.D. of virus (Barlow strain, 1 cc.). The animals all developed typical and, in most instances, fatal infections (Table III. Compare also Table IX, under the heading "Controls (heated virus alone).").

TABLE IV.

Effect of Injection of Neutral or Subneutral Mixtures of Virus and Immune Serum.

May 10, 1922. Virus: citrated guinea pig blood (Cooper strain), 1 cc. (100 M.L.D.).
Immune serum: rabbit.

Immune serum No.	Amount.	Vaccination reaction.	Immunity test.	
			Interval after vaccination.	Degree of protection.
	cc.			
11	0.1	Mild fever; recovery.	33 days.	Complete against 1,000 M.L.D.
			10 mos.	" " 30 "
	0.01	" " "	33 days.	" " 1,000 "
23	0.1	" " "	33 "	" " 1,000 "
	0.01	Typical infection; recovery.	33 "	" " 1,000 "
			8 mos.	" " 1 "
			9 "	" " 5 "
25	0.1	Mild fever; recovery.	33 days.	" " 1,000 "
	0.01	Moderate fever; recovery.		
186	0.1	Mild fever; recovery.	10 mos.	" " 30 "
	0.01	Moderate fever; recovery.		
307	0.1	Mild fever; recovery.	10 mos.	" " 30 "
	0.01	Typical infection; recovery.		
314	0.1	Mild fever; recovery.	3 mos.	" " 1,000 "
	0.01	Moderate fever; recovery.	3 "	" " 1,000 "
316	0.1	Mild fever; recovery.	8 "	" " 1 "
	0.01	Moderate fever; recovery.		
326	0.1	Mild fever; recovery.	6 mos.	" " 100 "
	0.01	Typical infection; recovery.		
327 (reinforced).	0.1	No reaction.	9 mos.	Complete against 5 M.L.D.
	0.01	Moderately severe infection.		

330 (reinforced).	0.1	No reaction.	33 days.	Complete against 1,000 M.L.D.
			10 mos.	" " 30 "
	0.01	Mild fever; died of intercurrent infection.		
341 (reinforced).	0.1	No reaction.	33 days	" " 1,000 "
			10 mos.	" " 30 "
	0.01	Moderate fever; recovery.		
350 (reinforced).	0.1	No reaction.	33 days.	" " 1,000 "
			10 mos.	" " 30 "
	0.01	Moderate fever; recovery.		

Titration of virus used for testing immunity. June 13, 1922.

Cooper strain virus.	Result of inoculation.
cc.	
0.1	Typical infection; death in 9 days.
0.01	" " " " 13 "
0.001	" " " " 11 "
0.0001	Mild fever; recovery.

Effect of Injection of Freshly Prepared Neutral or Superneutral Mixtures of Live Virus with Immune Serum.

It had been noticed early in these studies of immunity in spotted fever that guinea pigs which had received suitable mixtures of virus and immune serum remained well throughout several weeks of observation. The mixtures were innocuous, irrespective of the amount of virus introduced, provided the amount of immune serum were correspondingly adjusted. For example, 1 cc. of virus containing 100 or 1,000 M.L.D. might be injected without causing any infection when mixed with 0.1 cc. or more of immune serum, and animals so inoculated were found to be completely refractory to subsequent inoculation with as many as 100 or 1,000 M.L.D. The degree of immunity acquired was altogether comparable with that of animals which had received a subneutral mixture and had passed through a mild course of fever or a moderately severe infection with scrotal lesions. The immunity produced by subneutral mixtures is apparently the same as that following convalescence in animals which survive a typical severe infection caused by virus alone.

Table IV shows the neutralizing power of the serum of eight convalescent rabbits and of four hyperimmunized immune rabbits (*i.e.* which had received a second injection of virus during convalescence) in 0.1 and 0.01 cc. against 100 M.L.D. (Cooper strain, 1 cc.) and the degree of immunity developed in the guinea pigs. 0.1 cc. of the convalescent serum was an almost, but not completely, neutralizing dose, while the same quantity of the reinforced immune serums was sufficient to neutralize all infectivity. The guinea pigs were tested for immunity on several different occasions and with virus of various degrees of virulence. Of the eight animals tested 33 days later with 1,000 M.L.D., none became infected, and no change in temperature was observed. Two tested 3 months afterwards against 1,000 M.L.D. resisted infection. One animal was tested after 6 months with 100 M.L.D. and proved resistant. Several others were tested after longer periods—8, 9, and 10 months—against 1, 5, and 30 M.L.D. of virus and likewise proved immune.

Superneutral Mixtures.—In the case of human vaccination the neutral mixtures would leave too narrow a margin between the

neutral and the subneutral, and no margin can be too great to insure safety for the persons vaccinated. Since, however, a great excess of the immune serum in a mixture might reduce or even nullify the immunizing properties of the virus, it was necessary to determine this point. Hence a series of experiments was carried out in which a

TABLE V.

Effect of Injection of Superneutral Mixtures of Virus and Immune Serum.

May 25, 1922. Virus: citrated guinea pig blood (mixed Cooper and Barlow strains), 1 cc. (100 M.L.D.). Immune serum: rabbit.

Immune serum No.	Amount.	Vaccination reaction.	Immunity test.	
			Interval after vaccination.	Degree of protection.
	cc.			
11	1	Mild fever; recovery.	7 mos.	Complete against 1 M.L.D.
23	1	No reaction.	7 "	" " 1 "
25	1	" "	18 days.	Died of intercurrent infection; no lesions of spotted fever after injection of 1,000 M.L.D. virus.
186	1	" "	1 mo.	Complete against 100 M.L.D.
			4 mos.	" " 1,000 "
307	1	" "	1 mo.	" " 100 "
314	1	" "	4 mos.	" " 1,000 "
316	1	" "	4 "	" " 1,000 "
326	1	" "	4 "	" " 1,000 "
327 329	1	" "	Reserved for later test.	
330	1	" "	4 mos.	Complete against 1,000 M.L.D.
			7 "	" " 1 "
			7½ "	" " 5 "
332	1	" "	7½ "	" " 5 "
349	1	" "	7½ "	" " 5 "
350	1	" "	Reserved for later test.	

considerable excess of the serum was assured by using 1 cc. against 100 M.L.D. of virus. Fourteen different samples of immune serum, twelve of which been used also in the previous series of experiments, were used (Table V), all but one of which (No. 11) completely neutralized the virus. The animals were tested for immunity after 1, 4, 7, and 7½ months, respectively, and found to be completely

TABLE VI.

Effect of Various Quantities of Immune Serum on the Immunizing Power of the Virus.

June 24, 1922. Virus: citrated guinea pig blood (Barlow strain), 1 cc. (100 M.L.D. or 1,000 M.I.D.).
Immune serum: rabbit.

Immune serum No.	Amount.	Vaccination reaction.	Immunity test.	
			Interval after vaccination.	Degree of protection.
	cc.		*mos.*	
23	2	No reaction.	5½	Complete against 100 M.L.D.
			6	" " 1 "
			6½	" " 5 "
	2	" "	3	" " 1,000 "
	1	" "	3	" " 1,000 "
			7	" " 1 "
			7½	" " 5 "
	1	" "	3	" " 1,000 "
			7	" " 1 "
			7½	" " 5 "
	0.5	" "	7	" " 1 "
			7½	" " 5 "
	0.5	" "	Lost by intercurrent infection after 19 days.	
	0.25	Slight fever; recovery.	Reserved for later test.	
	0.25	Definite febrile reaction; recovery.	3	Complete against 1,000 M.L.D.
	0.1	Mild fever; recovery.	3	" " 1,000 "
	0.1	Severe fever; no scrotal lesions; recovery.	Reserved for later test.	
25	2	No reaction.	3	Complete against 1,000 M.L.D.
	2	" "	Reserved for later test.	
	1	" "	3	Complete against 1,000 M.L.D.
	1	" "	5½	" " 100 "

0.25	Some irregular rises of temperature, no spotted fever symptoms; recovery.	7½	Complete against	5 M.L.D.
0.25	No spotted fever symptoms.	Died of intercurrent infection in 23 days.		
0.1	Mild fever; recovery.	7	Complete against	1 M.L.D.
0.1	" " "	Reserved for later test.		

Titration of mixed virus (Cooper-Barlow) used for testing immunity. June 24, 1922.

Citrated guinea pig blood.	Results of inoculations.
cc.	
1	Typical severe infection with very marked scrotal lesions; died in 9 days.
0.1	Typical severe infection with very marked scrotal lesions; died in 10 days.
0.01	Typical severe infection with very marked scrotal lesions; died in 16 days.
0.001	Severe infection with extensive necrosis of scrotal skin, soles, and ear lobes; recovered.
0.0001	No infection.

resistant to infection with virus containing in some instances as many as 1,000 minimum lethal doses.

The results showed that the immune serum may be added to the virus in great excess without unfavorably influencing the development of immunity.

TABLE VII.

Duration of Passive Immunity.

June 24, 1922.

Immune serum (rabbit) No.	Amount.	Result.	Immunity test.
	cc.		
23	1	No reaction.	Tested 12 days later against Cooper virus 1 cc.;* typical infection; died in 9 days.
25	1	" "	Tested 12 days later against Cooper virus, 1 cc.;* mild infection; recovery.
			Tested 2 months later against Cooper virus, 1 cc. (1,000 M.L.D.); no infection.
	1	" "	Tested 2 months later against Cooper virus, 1 cc. (1,000 M.L.D.); typical severe infection; died in 11 days.

Titration of Cooper strain virus used for immunity tests. September 25, 1922.

Guinea pig citrate blood.	Result of inoculation.
cc.	
0.5	Severe infection with scrotal lesions; killed for virus in 10 days.
0.1	Severe infection with scrotal lesions; killed for virus in 10 days.
0.01	Severe infection; died in 13 days.
0.001	" " " " 13 "
0.0001	No infection.

* Control died in 14 days.

To confirm and supplement the above experiments, another series of animals was inoculated with mixtures containing the same amount of virus with varying amounts of immune serum. Two immune serums were separately used, and for each dose two animals were inoculated. 2, 1, 0.5, 0.25, and 0.1 cc. of serum, respectively, were mixed with 1 cc. of virus containing 100 minimum lethal doses or about 1,000 minimum infecting doses. No reaction followed the inoculation in the

animals which received the mixture containing 0.5 cc. or more of serum, but animals which received 0.25 cc. or less all had some degree of febrile reaction, although no scrotal lesions were noted. Irrespective of whether the quantity of serum was subneutral, neutral, or superneutral, all the animals proved to be equally resistant to several subsequent attempts to infect them after intervals of 3 months (with 1,000 M.L.D. of virus), 5, 5½, 6, and 7 months (with 1 to 5 M.L.D.

TABLE VIII.

Protective Power of the Blood of Vaccinated Guinea Pig.

Date of vaccination, May 25, 1922.

Material: virus (Cooper and Barlow strains mixed), 1 cc. + immune rabbit serum, 1 cc.

This animal belongs to the series reported in Table V.

Interval after vaccination.	Test of blood for protective power of citrate plasma.*	Result of inoculation.
days		
30	1 cc. + virus, 0.1 cc. (10 M.L.D. or 100 M.I.D.)	Definite febrile reaction; recovered.
	0.2 cc. + same virus, 0.1 cc.	Typical severe infection, with scrotal and ear lesions; recovered.
	0.1 " + " " 0.1 "	Typical severe infection; died in 10 days.

* 10 cc. heart blood were mixed with 10 cc. citrate solution; plasma collected after centrifugation

Of the fourteen other guinea pigs vaccinated on the same day with the same material, two were tested after the same interval (30 days) by injection of virus; both resisted infection with 100 M.L.D. of the same virus as used in the above experiment. The remaining twelve animals were tested at intervals of 4 to 7½ months with 1 to 1,000 M.L.D. of virus, and all resisted infection (see Table V).

of virus) (Table VI). It is evident, therefore, that the quantity of immune serum may be liberally increased if the mixture is to be used for vaccination.

The question arose whether the immunity which developed after the inoculations of neutral or superneutral mixtures might not be merely a passive immunity, due to the introduction of the immune serum. This point was decided by the experiment recorded in Table VII which showed that the inoculation of 1 cc. of a powerful

immune serum does not protect a guinea pig from subsequent infection 12 days or later; hence the immunity observed in guinea pigs 2 weeks or longer after the inoculation of neutral or superneutral mixtures cannot be ascribed to a passive immunity brought about by the antiserum introduced with the virus.

Protective Power of the Blood of Vaccinated Guinea Pigs.

From the theoretical point of view it was interesting to determine the neutralizing power of the blood of a guinea pig which had previously been inoculated with a neutral mixture of virus (Cooper-Barlow strains, 1 cc.) and immune serum (No. 28, 1 cc.). Blood was withdrawn from the heart 30 days after the time of vaccination. The citrated plasma was separated, and 1, 0.2, and 0.1 cc. were tested against 10 M.L.D. of the virus (Cooper strain, 0.1 cc.). The largest quantity, 1 cc., failed to neutralize the virus completely, but the animal recovered, while 0.2 and 0.1 cc. had no protective effect (Table VIII).

Fourteen other guinea pigs, vaccinated at the same time, and tested after the same period by injection of 100 M.L.D. of virus, all resisted the attempt to infect them. Hence the vaccinated guinea pigs are able to neutralize a far greater quantity of virus *in vivo* than the low neutralizing titer of their blood would lead us to expect.

Effect of Heating and of Age upon the Immunizing Properties of Neutral Mixtures of Virus and Immune Serum.

The strikingly effective protective value of a neutral or superneutral mixture of virus and immune serum against experimental spotted fever in guinea pigs suggested the possibility of applying the principle to human beings. The prospect of sero-vaccination, under the most rigid quantitative supervision of the virus and immune serum, is promising; nevertheless, it seems important, for the time being at least, to devise some method in which the virus in the mixture is killed, but which still gives a vaccine of definite protective value.

In Tables IX and X are recorded the results of experiments in which guinea pigs were inoculated with neutral or superneutral mixtures of virus and immune serum which had either been heated to 60°C. for

20 minutes in a water bath or preserved in the refrigerator (4°C.) for 32 days. One set of animals was inoculated with portions of the

TABLE IX.

Effect of Heating upon the Neutral Mixtures of Virus and Immune Serum.

October 5, 1922. Virus: citrated guinea pig blood, Cooper and Barlow strains (1 cc. contained 100 M.L.D.), 1 part. Immune serum: mixture of serum from several immune rabbits, 1 part.

Amount.	Result of vaccination.	Immunity test.		
		Interval after vaccination.	Tested against.	Degree of protection.
	Unheated.			
cc.				
2	No reaction.	18 days.	10,000 M.L.D.	Complete.
2	" "	18 "	10,000 "	"
2	" "	2 mos.	100 "	Died of secondary infection.
Controls (normal rabbit serum + virus).				
2	Typical infection; died in 11 days.			
2	Typical infection; died in 11 days.			
	Heated to 60° C. for 20 minutes.			
2	No reaction.	18 days.	10,000 M.L.D.	Mild fever; recovery.
2	" "	18 "	10,000 "	" " "
2	" "	2 mos.	100 "	Died of secondary infection.
	Controls (heated virus alone).			
2	No reaction.	18 days.	10,000 M.L.D.	Typical infection; died in 15 days.
1	" "	18 "	10,000 "	Typical infection; died in 12 days.
1	" "	2 mos.	100 "	Typical infection; died in 13 days.

freshly prepared mixture; another set received a portion of the same mixture, which had been previously heated; the remainder of the mixture was placed in the refrigerator and inoculated 32 days later.

Suitable controls (virus mixed with normal rabbit serum, and virus alone) accompanied each of the three sets of experiments.

TABLE X.

Effect of Age upon the Neutral Mixtures of Virus and Immune Serum.

Portions of the mixtures of virus and immune serum used on Oct. 5, 1922 (Table IX) were kept in the refrigerator at 4°C. until Nov. 6, 1922 (32 days).

Amount.	Result of vaccination.	Immunity test.		
		Interval after vaccination.	Tested against.	Degree of protection.
cc.				
2	No reaction.	16 days.	100 M.L.D.	Typical infection; died in 15 days.
2	" "	1 mo.	100 "	Irregular fever (secondary infection).
1	" "	16 days.	100 "	Suspicious febrile reaction but no lesions; died in 22 days of secondary infection.
1	" "	5 mos.	30 "	No infection.
	Controls (normal rabbit serum + virus).			
2	Secondary infection.			
1	No reaction.	16 days.	100 M.L.D.	Typical infection; died in 13 days.
	Controls (virus alone).			
2	No reaction.	1 mo.	100 M.L.D.	Typical infection; died in 19 days.
2	" "	16 days.	100 "	Typical infection; died in 14 days.
2	Suspicious febrile reaction; died in 16 days; spleen enlarged.			
1	No reaction.	1 mo.	100 "	Typical infection; died in 15 days.
1	" "	16 days.	100 "	Typical infection; died in 10 days.

Examination of the results shows, first that the mixture was superneutral, since there was no evidence of infection in any of the animals inoculated with the unheated fresh mixture. Two of these animals

were tested 18 days later against 10,000 M.L.D. of virus, the other 2 months later against 100 M.L.D., and both resisted infection. The latter animal died of intercurrent infection 3 weeks after the date of the immunity test. Normal rabbit serum had, of course, no neutralizing effect on the virus, and the two guinea pigs receiving the mixture of normal serum and virus died after 11 days.

Of the three animals which received the heated mixture of virus and immune serum, two, which were tested 18 days later against 10,000 M.L.D. of virus, had a mild febrile reaction but showed no lesions; the other, which was tested 2 months later against 100 M.L.D., died of intercurrent infection after 12 days; there were no specific pathological findings, and if the animal had a spotted fever infection, it must have been very mild. There is a great contrast between these results and those in the guinea pigs inoculated with the heated mixture of virus and normal rabbit serum, all of which died of typical infection.

Of the animals inoculated with the aged mixture, two died when tested 16 days later; one had a typical spotted fever infection, the other probably succumbed to secondary infection while recovering from mild spotted fever. The third, tested 1 month later, had irregular fever, but no symptoms of spotted fever. Of the animals which received the aged mixture of virus and normal rabbit serum, one died in 13 days of typical infection, the other was lost by intercurrent infection before the time of test. The aged virus alone produced neither infection nor immunity in the four animals receiving it; the two tested 16 days later died in 10 and 14 days, respectively, and the two tested after 1 month died in 19 and 15 days.

The contrast between the effect of fresh unheated and heated mixtures is again strikingly brought out in Table XI, in which are presented the results of the inoculation of smaller quantities of the superneutral mixture of virus and immune serum; one series of animals receiving respectively 1, 0.1, 0.01, and 0.001 cc. of the unheated, another the same quantities of the heated mixture. The injection of the heated mixture definitely modified the course of a subsequent test infection, although complete immunity was not induced even in a dose as large as 1 cc.; the unheated mixture conferred protection in a dose of 0.1 cc., and even in the animal receiving 0.01 cc. the infection was not fatal.

These findings confirm the earlier experiments establishing the effectiveness of fresh unheated neutral mixtures of virus and immune serum in producing immunity in guinea pigs. The uselessness of heated or aged virus, alone, or with normal rabbit serum, is definitely determined. The presence of a definite immunizing property in the

TABLE XI.

Effect of Superneutral Mixtures of Virus and Immune Serum in Various Amounts.

Virus: guinea pig serum, 1 cc., representing 1,000 M.L.D.

Immune serum: rabbit, 1 cc., representing ten times the quantity necessary to neutralize 1,000 M.L.D.

Amount.	Result of vaccination.	Result of immunity test, 34 days later, against 10 M. L. D. of virus.
Unheated.		
cc. 1 virus 500 M.L.D. + serum 0.5 cc.	No reaction.	No infection.
0.1 virus 50 M.L.D. + serum 0.05 cc.	" "	" "
0.01 virus 5 M.L.D. + serum 0.005 cc.	" "	Severe infection; recovery.
0.001 virus 0.5 M.L.D. + serum 0.0005 cc.	Definite mild febrile reaction.	No infection.
Heated.		
1	No reaction.	Mild infection; no lesions; recovery.
0.1	" "	Moderately severe infection; recovery.
0.01	" "	Severe infection; recovery.
0.001	" "	Typical infection; died after 14 days.

neutral mixture which had been heated to 56–60°C., and also, though in less degree, in the neutral mixture kept at 4°C. for 32 days, indicates the probable effectiveness of mixtures treated in this way for prophylactic vaccination in man, notwithstanding that these preparations fall much below the fresh mixtures in immunizing titer.

GENERAL CONSIDERATIONS.

From the findings outlined, it appears that the immunizing properties of the Rocky Mountain spotted fever virus are almost, if not totally, removed by heating to 60°C. for 20 minutes or by prolonged preservation at a low temperature. On the other hand, the immunizing power remains fully preserved when the virus is mixed with immune serum in neutral or superneutral proportions, notwithstanding the fact that such mixtures are completely devoid of infecting power. Moreover, heating of such mixtures to 56–60°C. for 20 minutes, or prolonged preservation at refrigerator temperature, does not altogether destroy, though it markedly reduces, the immunizing power. Since normal rabbit serum does not in any way influence or retard the rapid disappearance of immunizing properties from the virus under similar conditions (heating or aging), the persistence of immunizing power in the neutral or superneutral mixtures appears to be due to a specific reaction which takes place between the virus (antigen) and the immune serum (antibody).

For the purpose of inducing artificial immunity in man, the heated neutral mixtures of spotted fever virus and a highly potent immune serum may be used. The serum of infected guinea pigs is preferable to that of rabbits as the source of the virus, a high degree of virulence being more readily and uniformly maintained in the guinea pig. Immune serum from hyperimmunized rabbits is readily prepared and is capable of attaining a high degree of potency.[2] These two factors, highly virulent strains of the virus, and a powerful immune serum, are essential to the production of an effective sero-vaccine. The superneutral mixture is prepared as follows:

Blood is drawn from infected guinea pigs at the height of fever (4th to 5th day) and the clear serum separated from the clot.[3] The titer should be such that 1 cc. contains 100 M.L.D. of virus. By pooling a number of serums (after first making sterility tests of the individual serums to insure absence of secondary infection), virus of uniform

[3] It is imperative to determine by culture methods that the blood is free from any secondary bacterial infection, especially that caused by members of the paratyphoid group of bacilli, which induce febrile and splenic reactions strikingly similar to the symptoms shown by guinea pigs on the 4th and 5th days of spotted fever infection.

virulence can be obtained in large quantity. Immune rabbit serum, of a titer such that 0.1 cc. will neutralize 100 M.L.D., is mixed with the virus in ten times the neutralizing dose. A less potent immune serum is not suitable, because an inconveniently large quantity would be required. The mixture is heated to 56°C. for 20 minutes in a water bath and preserved in the refrigerator until used. Two or more injections of 1 cc. of the mixture may be given subcutaneously.

The procedure just outlined is a preliminary one only. The ultimate object of these experiments is a method in which the active (unheated) sero-vaccine may be safely applied to human vaccination.

SUMMARY.

Freshly prepared mixtures of spotted fever virus and immune rabbit serum in neutral or superneutral proportions confer complete immunity on guinea pigs.

The mixtures undergo a considerable loss in immunizing power when heated to 60°C. for 20 minutes, but are still capable, if used in sufficient quantity, of conferring a degree of immunity on the vaccinated animal such that a subsequent experimental infection is rendered less severe and non-fatal.

Unheated mixtures which had been preserved in the refrigerator at 4°C. for a period of 32 days still retained a certain degree of immunizing property.

The virus alone, or mixed with normal rabbit serum, when allowed to die out by prolonged preservation at refrigerator temperature, or when killed either by heating at 60°C. for 20 minutes or by chemicals (chloroform, ether, xylene) does not induce immunity in guinea pigs.

THE INFLUENCE OF THE FACTORS OF SEX, AGE, AND METHOD OF INOCULATION UPON THE COURSE OF EXPERIMENTAL SYPHILIS IN THE RABBIT.*

By ALAN M. CHESNEY, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research, New York, and the Syphilis Division of the Department of Medicine of Johns Hopkins University, Baltimore.)

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INTRODUCTION.

Our knowledge of the factors which govern the course of syphilitic infection in the rabbit is still incomplete. Until a systematic study has been made of those likely to influence the reaction of this experimental animal to infection with *Treponema pallidum*, our conceptions of the mechanism by which the infection is controlled are likely to be fragmentary at best. This communication deals with a study of some of the factors which seemed worthy of investigation from the standpoint of their influence upon the course of the syphilitic infection. The particular ones studied were those of age, sex, and method of inoculation.

Stated in more precise terms, the object was threefold: (1) to contrast the character of the disease in a group of females inoculated intradermally with that observed in a group of males inoculated in the same manner; (2) to contrast the character of the disease in a group of males inoculated intradermally with that in a group of males inoculated by the intratesticular route; and (3) to contrast the reaction of a group of young males following intratesticular inoculation with that of a group of older males. Opportunity was also afforded for a comparison of the course of the disease in males in which the primary focus following intratesticular injection had been removed by castration,

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with the course in animals in which no such attempt at interference with the ordinary progress of the disease had been made. In addition, it was possible to observe the course of the infection in animals in which attempts were made to produce chancres by implantation within the scrotum of bits of syphilitic rabbits' testicles. However, the influence of the factors of route of inoculation, age, and sex were the principal ones selected for study.

The experiment involved no new method of inoculation for the study of syphilis in the rabbit. All of the routes chosen have been used in the past, but there are not, to our knowledge, any published reports on a large series of animals inoculated simultaneously in various ways with the same material. So far as we are aware, the three factors studied in this experiment, age, sex, and method of inoculation, have not in the past been similarly studied on a given series of rabbits with identical infecting material. Experience with experimental syphilis in the rabbit has shown conclusively that a given series of animals inoculated at one time cannot be compared directly with a series inoculated at some other time, even when one is working with the same strain of *Treponema pallidum*. In any comparison of behavior of groups of animals toward infection with *Treponema pallidum*, it is essential that the various groups be inoculated at the same time, as well as with equal amounts of the same virus. Failure to observe this elementary rule will yield results which are of little value from a comparative standpoint in the study of experimental syphilis in the rabbit, hence the point is stressed here.

Technique.

The strain of *Treponema pallidum* used in these experiments was the Nichols strain isolated originally in 1912 from the spinal fluid of a case of neurorecidive (1). This strain, now nearly 11 years old, has had many passages through rabbits, and has attained a degree of virulence for that animal of such extent that it will produce primary lesions in 100 per cent, and under favorable conditions, will produce generalized lesions in from 80 to 100 per cent of animals inoculated intratesticularly, although the incidence of such lesions is at times very low. The immediate source of the material was testicular tissue of two rabbits that showed clinical evidence of orchitis 23 days after inoculation. The testicles were excised under ether anesthesia at a period when the syphilitic orchitis was progressing rapidly, portions of the body of the organ were finely minced with scissors, ground up in a sterile mortar with sterile salt solution, and the more fluid portion of the

resulting emulsion, which contained numerous actively motile organisms by dark-field examination, used for injection. Other parts of the body of the testicle were cut into small bits, 2 to 3 mm. in thickness, and these pieces served for scrotal implantation.

Intradermal Inoculation.—This method was accomplished by inserting the needle in the subcutaneous tissue of the sheath of the penis or vulva, at a distance of about 1 cm. from the mucocutaneous border, directing the needle toward the latter and at the same time bringing the top of the needle (bevel up) toward the surface until it lay in the skin just beneath the superficial layer, where it could be easily seen, and carrying it as far as the mucocutaneous border.

Intratesticular Inoculation.—This method needs no description.

Scrotal Implantation.—This method has already been described (2) and need not be discussed at length.

In the case of the intradermal and intratesticular inoculations the same amount of emulsion was used throughout; namely, 0.1 cc. For obvious reasons, the scrotal implants could not be of exactly the same size, but were approximately so.

The animals belonged to a variety of breeds and were kept under observation for a period of 90 days. The selection of this period was based upon the course of events following a unilateral testicular inoculation, since experience with the strain had shown that within this interval the infection would pass through a more or less orderly evolution of primary and secondary lesions, while observation over longer periods would entail the possibility of confusion through the occurrence of relapses. Hence, this period was selected as the most suitable for the comparisons that we had in mind. No attempt was made to demonstrate treponemata in the lesions as they appeared because of the possibility of the trauma exerting an influence on the course of the infection.

In all, thirty-seven rabbits were inoculated, of which number two died from pulmonary disease during the course of the experiment and are excluded from consideration. The remaining thirty-five which are to be reported may be divided into six groups as follows: (A) five adult females (over 12 months) inoculated intradermally; (B) seven adult males (over 12 months) inoculated intradermally; (C) five young males (below 5 months) inoculated intratesticularly; (D) seven adult males inoculated intratesticularly; (E) five adult males inoculated intratesticularly, castrated; (F) six adult males inoculated by scrotal implantation. The inoculations were all made on October 5, 1922.

EXPERIMENTAL.

Unless expressly stated otherwise in the ensuing protocols it will be understood that all primary lesions developed at the site of inoculation.

Group A.

This group comprised five adult females. Each animal received 0.1 cc. of testicular emulsion intradermally at the mucocutaneous border of the vulva.

Rabbit No. 1.—New Zealand red. No lesions of any sort except slight inguinal and popliteal lymphadenopathy.

Rabbit No. 2.—Brownish grey. After 11 days an infiltrated area, 3.0 mm. in diameter, and inguinal adenopathy. Maximum diameter, 4.0 mm., attained on the 15th day, later disappearance, followed by two recurrences on the 40th day.

Rabbit No. 3.—Brown. Infiltrated area on the 10th day, 1.0 mm. in diameter, disappearing on the 12th day. Slight inguinal lymphadenopathy. No recurrence.

Rabbit No. 4.—Brown. Infiltrated area appeared on the 19th day, increased to 1 cm. in diameter on the 52nd day, and ulceration occurred, then the lesion gradually receded. Inguinal and popliteal lymphadenopathy.

Rabbit No. 5.—Black. Indurated area, 3.0 mm. in diameter on the 10th day, reached maximum diameter of 8.0 mm. on the 21st day, then diminished gradually. Inguinal and popliteal lymphadenopathy.

Recapitulation.—Of the five animals in this group, one animal showed no local lesion save the initial reaction following injection, while another showed a small infiltrated area which disappeared within 10 days. In a third animal the local lesion consisted at the most of a papule, 4 mm. in diameter, and in a fourth, the lesion consisted of a papule attaining a maximum diameter of 8 mm. In only one of the group did the lesion develop into a characteristic chancre with necrosis of the epithelium, and ulceration. Not one of the animals showed the presence of generalized lesions (bone, skin, eye) within the period of 90 days following injection. In this group, then, the disease in general manifested itself as an inconstant and relatively mild local reaction without clinical evidence of generalization, except for a comparatively slight and rather uncertain reaction in the inguinal and popliteal lymph nodes.

Because of the absence of any generalized lesions in this group it seemed worth while to determine, by transfer of lymph node material to normal rabbits' testicles, if the organisms had invaded the body in spite of the slight local reaction at the inoculation site. The popliteal lymph nodes were removed from each animal in the group and, after

emulsification in salt solution, were injected into the testicles of two normal rabbits, respectively. Of the ten animals to which node transfers were thus made, all developed an orchitis containing treponemata within a period of not more than 62 days after transfer, demonstrating that in all of the five females, infection and general dissemination had taken place in spite of the relatively slight degree or total absence of lesions at the site of inoculation, and in spite of the failure of production of clinically recognizable generalized lesions.[1]

Group B.

This group consisted of seven adult males. Each animal received 0.1 cc. of testicular emulsion intradermally in the sheath of the penis. The individual protocols follow.

Rabbit No. 6.—Brownish grey. Infiltrated area, 1 mm. in diameter, 31st day; disappeared on the 42nd day. Transient and immediate inguinal lymphadenopathy. No popliteal lymphadenopathy.

Rabbit No. 7.—Brown. Papule, 2.0 mm. in diameter, on the 31st day; on the 61st day three papules appeared and increased in size during the period of observation. Right-sided orchitis on the 89th day. Inguinal adenitis developed early.

Rabbit No. 8.—New Zealand red. Infiltrated area, 2.0 mm. in diameter, appeared on the 10th day, increased in size, developed into a chancre with a maximum diameter of 8.0 mm. on the 74th day. Inguinal and popliteal lymphadenopathy.

Rabbit No. 9.—Albino. Infiltrated area appeared on the 6th day, increased in size, and became a typical chancre with maximum diameter of 1.5 cm. on the 74th day. Left-sided periorchitis on the 85th day. Inguinal and popliteal lymphadenopathy.

Rabbit No. 10.—Brown. Infiltrated area appeared on the 10th day, became a typical chancre with a maximum diameter of 1.8 cm., and was increasing in size at the end of the experiment. Skin papule on right foot on the 84th day, bilateral orchitis at same time. Skin lesion on the left foot and lesions of the left tarsus, the left fifth metatarsal, and the left ulna on the 89th day.

Rabbit No. 11.—Brown. Infiltrated area appeared on the 10th day, regressed, then became a chancre with a maximum diameter of 10 mm. on the 61st day. Inguinal and popliteal lymphadenopathy.

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[1] This portion of the work was carried out in the Syphilis Division of the Department of Medicine of the Johns Hopkins Medical School, and was participated in by Dr. Jarold E. Kemp, to whom thanks are due.

Rabbit No. 12.—Grey. Infiltrated area appeared on the 10th day, a maximum diameter of 5.0 mm. attained on the 19th day; the lesion then receded. No ulceration. Transient inguinal lymphadenopathy.

Recapitulation.—In this group of seven animals there was one in which the only local lesion was a transient tiny area of infiltration, a second in which the local lesion developed into an indurated area, 0.5 cm. in diameter at its maximum, and a third in which the local lesion consisted of three infiltrated papules. In this latter animal evidence of generalization of the virus was provided by an orchitis appearing on the 89th day following injection. In the remaining four animals characteristic chancres appeared on the prepuce at the site of inoculation, and in two of these animals generalized lesions occurred. Summarizing these results: one animal showed only a slight and transient local lesion; two showed persistent lesions which were in the nature of papules which did not ulcerate; and four developed typical chancres. Generalized lesions, consisting of metastatic orchitis, skin papules, or periosteal thickenings, developed in three of the animals within a period of 90 days; the lesions of skin or bones, however, occurred in only one animal of the group. In this group the primary reaction at the site of inoculation was much greater than in the group of females, and the regional lymphadenitis was more marked. Exclusive of testicular involvement, one of the seven rabbits showed generalized lesions, while none of the females showed such lesions.

Group C.

This group consisted of five young males, all less than 6 months of age. Each received 0.1 cc. of testicular emulsion containing spirochetes, in the right testicle. The individual protocols follow.

Rabbit No. 13.—Brown. Orchitis (right) appeared on the 10th day, scrotal edema on the 24th day, metastatic orchitis (left) on the 46th day, and a scrotal chancre (right) on the 60th day. Skin lesion appeared on the right foot on the 66th day.

Rabbit No. 14.—Brown. Nodular orchitis (right) appeared on the 17th day, scrotal edema on the 24th day, and metastatic orchitis (left) on the 40th day. Skin lesion appeared on the right foot on the 62nd day, and a similar lesion on the left foot on the 73rd day.

Rabbit No. 15.—Brown. Orchitis (right) appeared on the 10th day, a scrotal edema on the 19th day, metastatic orchitis (left) on the 46th day, and a scrotal chancre (right) on the 60th day. Skin lesion appeared on the left foot on the 60th day.

Rabbit No. 16.—Brownish grey. Orchitis (right) appeared on the 10th day; the entire testicle involved on the 24th day, after which the orchitis regressed, and no other lesions appeared.

Rabbit No. 17.—Greyish brown. Orchitis (right) appeared on the 17th day, scrotal edema on the 25th day, and metastatic orchitis (left) on the 48th day. Scrotal chancre (right) appeared on the 52nd day and skin lesions appeared on the right foot on the 70th day. A second scrotal chancre appeared on the 77th day on the right side.

Recapitulation.—Of the five young males injected intratesticularly an initial orchitis occurred in all. In four, or 80 per cent, an orchitis developed in the non-inoculated testicle within an average period of 43.7 days. All of these four animals showed skin lesions within an average period of 64 days. One animal only failed to develop metastatic lesions in the testicle or elsewhere.

The outstanding feature of the condition presented by this group of animals was the occurrence of severe primary lesions and of well developed lesions in the uninoculated testicle, as contrasted with comparatively slight lesions elsewhere, all of which were confined to the skin.

Group D.

This group comprised seven adult males inoculated intratesticularly with 0.1 cc. of testicular emulsion containing treponemata. Only one testicle was inoculated and no attempt was made to interfere with the course of the infection. The individual protocols follow.

Rabbit No. 18.—Himalaya cross. Orchitis (right) appeared on the 10th day, a scrotal chancre (right) on the 42nd day, and metastatic periorchitis on the 46th day. Skin papule appeared on the right foot on the 62nd day.

Rabbit No. 19.—Grey. Orchitis (right) appeared on the 17th day, scrotal edema (right) on the 26th day, followed by massive nodular orchitis. No metastatic lesions were observed.

Rabbit No. 20.—Flemish cross. Orchitis (right) appeared on the 19th day, scrotal edema (right) on the 29th day, metastatic orchitis (left) on the 35th day, and a scrotal chancre (right) on the 66th day. A skin lesion appeared on the right foot on the 49th day. Scrotal chancre appeared on the left side on the 84th day and a skin lesion appeared on the left foot at the same time.

Rabbit No. 21.—Belgian cross. Orchitis (right) appeared on the 10th day, scrotal edema (right) on the 17th day, and metastatic orchitis (left) on the 38th day. Skin lesions appeared on the left foot on the 49th and 60th days. Scrotal chancre (right) developed on the 66th day.

Rabbit No. 22.—Black and Flemish cross. Orchitis (right) appeared on the 10th day, scrotal edema (right) on the 21st day, and metastatic orchitis (left) on the 38th day. Skin lesions appeared on the right foot on the 56th day, and on the left foot on the 64th day; periosteal lesion of fifth left metatarsal on the 60th day. Phlyctenular lesions appeared on the sclerocorneal margin of the left eye at the same time.

Rabbit No. 23.—Greyish brown. Orchitis (right) appeared on the 10th day, scrotal edema (right) on the 27th day, and metastatic orchitis (left) on the 46th day. No other generalized lesions occurred.

Rabbit No. 24.—Brown. Orchitis (right) appeared on the 17th day, scrotal edema on the 19th day, and metastatic orchitis (left) on the 54th day. Skin lesions appeared on the left foot on the 49th and 53rd days, on the right foot on the 55th day, and at the base of the tail on the 60th day. Fusiform swelling of the shaft of the fifth right metatarsal appeared on the 45th day.

Recapitulation.—Of the seven adult males which were inoculated intratesticularly, six, or 85.7 per cent, showed metastatic orchitis within a period of 41.5 days, and five, or 71.4 per cent, had metastatic lesions of other structures (skin and bone) within an average time interval of 54.7 days.

Group E.

This group comprised five adult males which were inoculated intratesticularly with 0.1 cc. of testicular emulsion containing treponemata. Only one testicle was inoculated. On the 11th day following inoculation, when lesions could be detected by clinical examination in all of the animals, the entire right testicle was removed under ether anesthesia and the wound closed with sutures. The individual protocols follow.

Rabbit No. 25.—Albino. Orchitis (right) appeared on the 10th day. Castration (right) on the 11th day. Recurrent lesion of the stump on the 38th day, later becoming a chancre; metastatic epididymitis (left) on the 53rd day. Skin lesions appeared on the right foot on the 84th day and on the left foot on the 90th day.

Rabbit No. 26.—Brown and Maltese cross. Orchitis (right) appeared on the 10th day. Castration (right) on the 11th day. Recurrent lesion of stump on the 38th day, later developing into a chancre. Metastatic orchitis (left) on the 40th day. No other generalized lesions were noted.

Rabbit No. 27.—Orchitis (right) appeared on the 10th day. Castration (right) on the 11th day. Some induration of the stump appeared but no marked lesion developed. Metastatic orchitis (left) appeared on the 50th day, lesion of nasal bones on the 70th day, and a skin lesion of the right foot on the 85th day.

Rabbit No. 28.—New Zealand red. Orchitis (right) appeared on the 10th day. Castration (right) on the 11th day. No metastatic lesions of any sort were observed, nor were there any recurrences at the operative site.

Rabbit No. 29.—Brown. Orchitis (right) appeared on the 10th day. Castration (right) on the 11th day. Recurrence at the operative site on the 29th day. Metastatic orchitis (left) on the 42nd day. Periosteal bone lesions appeared on the fifth right metatarsal on the 52nd day, on the left ulna on the 53rd day, and on the nasal bones on the 63rd day.

Recapitulation.—Of the five adult males which were inoculated intratesticularly and the primary focus removed later by castration, four, or 80 per cent, showed a metastatic orchitis within a period of 46.7 days, and in three, or 60 per cent, metastatic skin or bone lesions made their appearance within 90 days. In this group the metastatic lesions were slower in making their appearance than in the control group (Group D), in which there was no attempt made to interfere with the course of the primary focus. The ultimate incidence of generalized lesions was approximately the same in the two groups. In three of the five castrated animals, pronounced recurrent lesions developed in the operative wound. In a fourth there was a similar although less marked recurrence.

Group F.

This group comprised six adult males. All of the animals in the group were inoculated by implantation, within the right scrotal sac, of bits of syphilitic testicular material containing treponemata. The individual protocols follow.

Rabbit No. 30.—Grey. Scrotal chancre appeared on the 10th day, orchitis (right) was detected on the 31st day. Skin lesions appeared on the left foot on the 49th day, and on the right foot on the 60th day. Periosteal lesions of both radii were observed on the 52nd day, and of the right ulna on the 66th day. The chancre began to heal spontaneously before the 90th day.

Rabbit No. 31.—Albino. Scrotal chancre developed by the 10th day, a skin lesion appeared on the left foot on the 70th day. The chancre healed spontaneously before the 90th day.

Rabbit No. 32.—Albino. Scrotal chancre developed on the 10th day. Metastatic orchitis (left) appeared on the 52nd day, and on the 60th day a subcutaneous nodule of uncertain nature appeared. The chancre, after attaining an enormous size, began to heal before the 90th day.

Rabbit No. 33.—Brown. Scrotal chancre developed by the 10th day; skin lesions appeared on both feet. The chancre healed spontaneously before the 85th day.

Rabbit No. 34.—Brown. Two scrotal chancres developed by the 17th day, one at the site of implant, the other at the scrotal incision. Metastatic epididymitis (left) was noted on the 64th day. No other metastatic lesions were observed, while both chancres healed spontaneously by the 66th day.

Rabbit No. 35.—Belgian cross. Two scrotal chancres developed by the 10th day, one at the site of the implant, the other at the scrotal incision. They coalesced, forming a single chancre by the 20th day. Metastatic orchitis (left) appeared on the 46th day, skin lesions on the left foot on the 49th day, and on the right foot on the 60th day. A lesion of the nasal bones was observed on the 60th day. The chancre, after regressing appreciably, relapsed and was increasing in size at the end of the experiment.

Recapitulation.—Of the six adult males which received implantations of syphilitic testicular material in the scrotum, all developed typical chancres which healed spontaneously. In three, or 50 per cent, metastatic orchitis or epididymitis made its appearance within an average time interval of 54.9 days; and five, or 83 per cent, showed metastatic lesions of other structures in an average of 51.6 days.

GENERAL SUMMARY.

For convenience the results are summarized in tabular form. Table I shows a comparison of the primary reaction in the groups of female and male rabbits inoculated intradermally. In Table II the incidence of metastatic lesions is shown in the various groups of animals. In Table III are shown the character and date of appearance of the generalized lesions (exclusive of orchitis) in the various groups of animals.

The results presented in Tables I, II, and III, together with a study of the individual protocols, permit the following general statements to be made relative to the effect of the factors studied upon the course of experimental syphilis in the rabbit.

Sex.—In the group of females inoculated intradermally the lesions at the site of inoculation were in general less marked than in the group of males similarly inoculated. Moreover, they attained their maxi-

mum size earlier and began to recede earlier than was the case in the males. In none of the females was there clinical evidence of the production of generalized lesions, while in the corresponding group of males metastatic lesions of skin or bones detectible by clinical examination occurred in one instance, or 14.3 per cent. In both groups there was wide variation in the character of the initial reaction in individual rabbits and in the time required for it to reach its maximum size.

TABLE I.

Comparison of Primary Reactions in Male and Female Rabbits Inoculated Intradermally with Treponema pallidum.

Group.	Rabbit No.	Character of primary lesion.	Incubation period.	Maximum diameter of primary lesion.	Time of appearance of maximum size of primary lesion.
			days	*mm.*	*days*
A.	1	None.			
5 females.	2	Indurated papule.	11	4	15
	3	Transient indurated area.	10	1	10
	4	Chancre.	19	10	52
	5	Indurated papule.	10	8	21
Average			10	4	19.6
B.	6	Transient indurated area.	31	1	31
7 males.	7	Three indurated papules.	31	5	?*
	8	Chancre.	10	8	74
	9	"	6	15	74
	10	"	10	18	?*
	11	"	10	10	61
	12	Indurated papule.	10	5	19
Average			15.4	9	

* Lesion still increasing in size at the end of the experiment.

Age.—In the five young males inoculated intratesticularly the disease was not greatly different from that observed in the older males similarly inoculated. The initial reactions were slightly slower in making their appearance, but the magnitude of the reaction, as judged by enlargement of the testicles, was somewhat greater. The percentage incidence of metastatic orchitis was almost the same in the two groups. The younger animals showed a slightly greater incidence of

generalized lesions involving structures other than the testicles, although the difference was insignificant, but the lesions were smaller, fewer in number, and confined entirely to the skin, and there was no instance of severe generalized lesions. Moreover, the occurrence of metastatic lesions (skin) in the younger animals was definitely delayed.

Method of Injection.—In the animals inoculated by the intratesticular route the development of the primary reaction and the incidence of generalized lesions involving skin and bone were much greater than in

TABLE II.

Incidence of Metastatic Syphilitic Lesions in Various Groups of Rabbits.

Group and method of inoculation.	No. inoculated.	Metastatic orchitis.			Generalized lesions (bone, skin, eye).		
		No.	Per cent.	Average time of appearance.	No.	Per cent.	Average time of appearance.
Intradermal inoculation.							
				days			*days*
A. Adult females.	5				0	0	
B. Adult males.	7	3	42.8	87	1	14.3	84
Intratesticular inoculation.							
C. Young males.	5	4	80	43.7	4	80	64
D. Adult males, not castrated.	7	6	85.7	41.5	5	71.4	54.7
E. " " castrated.	5	4	80	46.7	3	60	69
Scrotal implantation.							
F. Adult males.	6	3	50	54.9	3	50	51.6

the animals inoculated intradermally. Thus, of the latter group only one, or 14.3 per cent, showed a secondary metastatic lesion (skin), while in the group inoculated by the testicular route the incidence of metastatic skin and bone lesions was as high as 71.4 per cent. The difference in the character of the disease exhibited by these two groups of animals inoculated in a different manner is quite striking. Intratesticular inoculation produced a much more violent local reaction and a much greater incidence of generalized lesions than did intradermal inoculation.

Effect of Castration.—In the group of animals with unilateral orchitis in which the infection was allowed to run its course without any attempt at suppression by removal of the initial focus, metastatic lesions appeared more promptly and in slightly greater incidence than

TABLE III.

Character and Date of Appearance of Generalized Syphilitic Lesions (Exclusive of Orchitis) in Various Groups of Rabbits.

Group.	Rabbit No.	Generalized syphilis (exclusive of orchitis and lymphadenitis).	Lesions in order of their appearance.	Recurrence.
Intradermal inoculation.				
			days	
A. 5 females.	1	—		
	2	—		
	3	—		
	4	—		
	5	—		
B. 7 males.	6	—		
	7	—		
	8	—		
	9	—		
	10	+++	S(89), PB(89), PB(89)	
	11	—		
	12	—		
Unilateral testicular inoculation.				
C. 5 young males.	13	+	S(66)	
	14	++	S(62, 73)	
	15	+	S(60)	
	16	—		
	17	+	S(70)	
D. 7 males.	18	+	S(62)	
	19	—		
	20	++	S(49, 84)	
	21	++	S(49, 60)	
	22	++++	S(56), PB(60), E(60), S(64)	
	23	—		
	24	+++++	S(49), PB(52), S(53, 55, 60)	

E indicates eyes; S, skin; PB, periosteum and bone.

TABLE III—*Concluded.*

Group.	Rabbit No.	Generalized syphilis (exclusive of orchitis and lymphadenitis).	Lesions in order of their appearance.	Recurrence.
Unilateral testicular inoculation, castration.				
E.	25	++	S(84, 90)	+
5 males.	26	—		+
	27	++	PB(70), S(85)	±
	28	—		—
	29	+++	PB(52, 53, 63)	+
Scrotal implantation.				
F.	30	++++	S(46), PB(52), S(60), PB(66)	
6 males.	31	+	S(70)	
	32	±	S(60)?	
	33	+	S(60)	
	34	—		
	35	+++	S(49), PB(60), S(60)	

in the group of animals in which the initial local reaction was suppressed by removal of the focus itself through castration. The percentage differences are not very great, but they are constantly in favor of the first group, as is the time interval of appearance. This experiment is not in strict accord with similar experiments observed by Brown and Pearce (3) in the past, and while the reasons for this difference are not clear the fact may be noted that the incidence of generalized lesions in uncastrated animals is somewhat higher than that reported by these authors, also that there was a high percentage of recurrence of primary lesions.

DISCUSSION.

The results are sufficiently clear-cut to permit of a generalization on several points. The difference in the character of the initial local response and in the subsequent production of generalized lesions, exhibited by the methods of intradermal and intratesticular inoculation, is striking, and can scarcely be explained upon the basis of biological variation in groups of animals. One is forced to conclude either that the superficial layers of the skin of the rabbit (the scrotum excluded) constitute a relatively unfavorable site for the active proliferation of

the treponemata when small amounts are introduced, or else that the defensive mechanism at this spot is brought to such a level as almost completely to suppress the initial reaction, without, however, preventing general invasion of the body with lymph gland involvement. By contrast the testicle is a much more favorable site for the setting up of a clinically recognizable lesion. Whether this is due to the presence in the testicle of an abundance of substances favoring growth of the treponemata, or to the absence of conditions that favor the operation of factors of resistance it is impossible to say. Perhaps both influences may be at work side by side. The successful production of primary reactions in the scrotum by implantation of bits of tissue containing treponemata does not in our opinion alter the generalization that the primary reactions obtained by testicular inoculation are greater than those obtained by intradermal inoculation. In the case of the scrotal implantations the inoculum was of necessity much larger than that employed for intratesticular and intradermal inoculation.

The difference in the behavior of the females and the males toward intradermal inoculation is of interest. In the former the local reaction was much less prominent than in the males, attained its maximum size more rapidly, and cleared up more promptly. None of the five females developed generalized lesions of other structures, whereas in two of the seven males an orchitis developed, and in a third there was evidence of generalization of the virus in the shape of skin and bone lesions. The behavior of these five females was different from that of eight normal females formerly reported in a paper (4) dealing with the reaction of pregnant females to inoculation with *Treponema pallidum*. In that paper it was stated that non-pregnant female rabbits reacted to intradermal inoculations of the vulva with the prompt development of characteristic indurated lesions at the site of inoculation. Pregnant animals, however, when inoculated under similar conditions were found to react either not at all or with much less marked clinical phenomena. In the five non-pregnant animals reported upon in this paper the reaction to intradermal inoculation approached more closely that exhibited by the pregnant animals in the foregoing paper. This discrepancy in results is probably explainable on the basis of a difference in virulence or a difference in the severity of the prevailing disease since a difference of the same character was observed in the behavior of the male animals of the two series.

Whatever its explanation, it serves to show that the behavior of any one group of rabbits toward infection with *Treponema pallidum* is by no means indicative of what may be expected subsequently in another group of animals even if infected with the same strain. In our opinion this point has not received sufficient attention, particularly from some of those workers in experimental syphilis who have been attempting to classify strains on the basis of the primary reactions exhibited by the rabbit host. Many of the variations in primary reactions that have been proposed as characteristics for differentiating one strain from another were, to judge from their description, no more marked than those exhibited by the group of female rabbits reported upon in this paper.

It is of interest, also, that in all of the five females that were inoculated intradermally the organisms were able to invade the body and localize in distant lymph nodes in spite of the relatively slight local lesion at the portal of entry or the total absence of the same. This observation parallels similar observations by Neisser in monkeys and is in agreement with previous work (5) in which it was demonstrated that *Treponema pallidum* might make its way through the genital mucosa of rabbits and invade the body, without calling forth any reaction at the site of inoculation.

With respect to the factor of age, the results were not sufficiently clear-cut to warrant any broad generalization. In general, the younger animals reacted with a more violent initial lesion, and were apparently able to keep the subsequent manifestations under control for a slightly longer period of time, as well as to prevent the occurrence of any severe generalized lesions. There was a rather striking uniformity in the manner in which the individual animals in this group reacted to the infection at the site of inoculation.

The effect of removal of the initial focus on the subsequent production of generalized lesions, which in former experiments by Brown and Pearce (3) resulted in a greater incidence of the latter, was not observed in the experiments herein reported. In those animals in which the primary reaction was removed by castration the incidence of generalized lesions was no greater and the time of occurrence was distinctly postponed. The former experiments seemed to point definitely to an influence on the course of the infection exerted by the initial lesion. Procedures calculated to suppress the initial lesion (removal, insufficient treatment with arsenicals) resulted in a greater incidence,

within certain time limits, of generalized lesions in the animals in which the interference with the initial lesion was instituted than in the controls. Upon this basis an hypothesis spoken of as a "law of inverse ratio" was formulated. The experiments herein described would seem to indicate that under conditions as yet unknown there may be exceptions to this principle.

CONCLUSIONS.

1. Rabbits inoculated intradermally with active syphilitic virus are within specified time limits less prone to develop generalized syphilis recognizable by clinical methods of examination than those inoculated intratesticularly.

2. The primary reaction to intradermal inoculation of syphilitic virus was distinctly less marked in females than in males.

3. Young rabbits react to an intratesticular inoculation of the syphilitic virus with a slightly more marked initial lesion than do older animals similarly inoculated, and tend to postpone slightly the appearance of subsequent generalized lesions, which are less severe in character and extent.

4. Suppression of the initial reaction by excision does not always lead to an increased incidence or early appearance of subsequent generalized lesions within an observation period of 90 days.

5. In rabbits inoculated intradermally in which no reaction or only a slight reaction developed at the site of inoculation, general invasion of the body by the treponemata may take place, as evidenced by positive lymph node transfer, although no generalized lesions may be observable.

The experiments reported in this paper were carried out at the suggestion and under the supervision of Dr. Wade H. Brown. Grateful acknowledgement is hereby made to Dr. Brown and to Dr. Louise Pearce for their interest and assistance in prosecuting the work.

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LOCAL RESISTANCE TO SPONTANEOUS MOUSE CANCER INDUCED BY X-RAYS.

By JAMES B. MURPHY, M.D., JOSEPH MAISIN, M.D., and ERNEST STURM.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

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Recent experimentation has tended to show that cancer is not so sensitive to radiation as earlier observers believed.[1] Although there may be some variation in the amount of x-rays required to kill various transplanted mouse[2] and rat tumors, the more careful experiments indicate that the lethal dose is rarely, if ever, within the limits of a therapeutic dose for man. On the other hand, there is no doubt that certain forms of cancer are cured by x-rays.

The recent revival of interest in x-ray therapy, due largely to the development of apparatus for generating more penetrating rays, opens up anew the question of the mode of action of this agent. If cancer is more sensitive to x-rays than normal tissue, as is generally believed to be the case, this new development is unquestionably a move in the right direction; but there is no substantial experimental basis to uphold this belief, and very good evidence to the contrary. Obviously the two facts—that cancer cells are not easily killed by x-rays, and yet that cancer may be cured by this agent—require examination if x-ray therapy is to be put on a rational basis and to be developed into a more effective form of treatment.

It has already been shown that x-rays, given over an area of skin in an erythema dose, render this area highly resistant to a subsequent inoculation with a transplantable cancer.[3] It is our opinion that this increased resistance is due to the fact that x-rays induce in this

[1] Hill, E., Morton, J. J., and Witherbee, W. D., *J. Exp. Med.*, 1919, xxix, 89.

[2] Wood, F. C., and Prime, F., *J. Am. Med. Assn.*, 1920, lxxiv, 308.

[3] Murphy, Jas. B., Hussey, R. G., Nakahara, W., and Sturm, E., *J. Exp. Med.*, 1921, xxxiii, 299.

exposed area a pronounced cellular reaction of the type which under other conditions is associated with resistance to cancer. As a transplantable tumor was used for these experiments, no general deduction can be drawn as to the behavior of the spontaneous disease, and therefore it seemed advisable to determine whether the same principle was operative under the same experimental conditions when autografts of spontaneous cancer were used, thus reproducing conditions more nearly comparable to those which exist for the disease as it occurs in man.

Experiment 1.—A mouse with a spontaneous mammary cancer was operated upon with removal of the tumor. With the tumor out, an area on the left flank, 12 x 15 mm. was exposed to an erythema dose of x-rays, governed by the following facts: spark-gap 3 inches; milliamperes 10; distance 6 inches; time 2½ minutes. Immediately after this treatment a small bit of the original cancer was reinoculated intracutaneously in the x-rayed area, and a like graft in the right flank, which had been protected from the x-rays (Text-fig. 1).

Among forty-nine mice with various types and stages of mammary tumors, subjected to this treatment, the graft inoculated in the x-rayed area failed to grow in thirty-five of the animals (71.4 per cent), while the graft in the untreated area failed in only eight (16.4 per cent). When the graft in the x-rayed area grew, it invariably progressed at a much slower rate than the corresponding graft in the normal skin, so that at the time of death of the animal it was never more than a fraction of the size of the other tumors (Text-fig. 2).

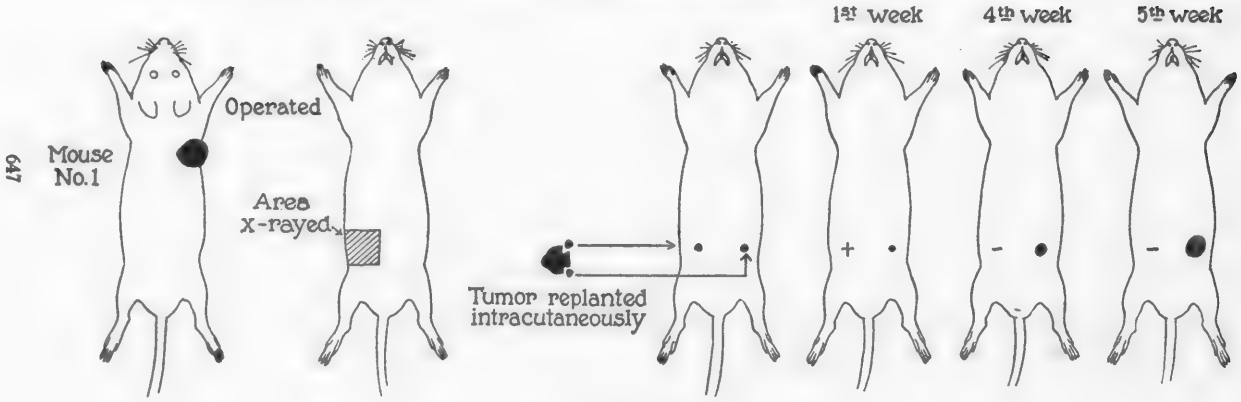
From these experiments it is evident that the local immunizing power of x-rays is just as effective against autografts of spontaneous cancers as it is against implants of a transplantable tumor. In this experiment, however, as well as in the earlier ones dealing with the transplantable tumor, the x-ray treatment at best has prevented a take or retarded the subsequent growth of the graft. It is conceivable that slightly unfavorable environmental conditions, insufficient to influence an established tumor, might be sufficient to prevent the take of a graft in which presumably the tumor cells are at a disadvantage. Will the conditions induced by x-rays be sufficiently unfavorable to influence an established tumor in the skin? The following experiment was outlined to answer this question, and also, by way of comparison, to test the direct action of x-rays on the cancer cells.

Experiment 2.—A mouse with spontaneous cancer was operated on with removal of the tumor. The tumor was then divided into two parts, and one of these subjected to an erythema dose of x-rays *in vitro*. (Spark-gap 3 inches; mili-

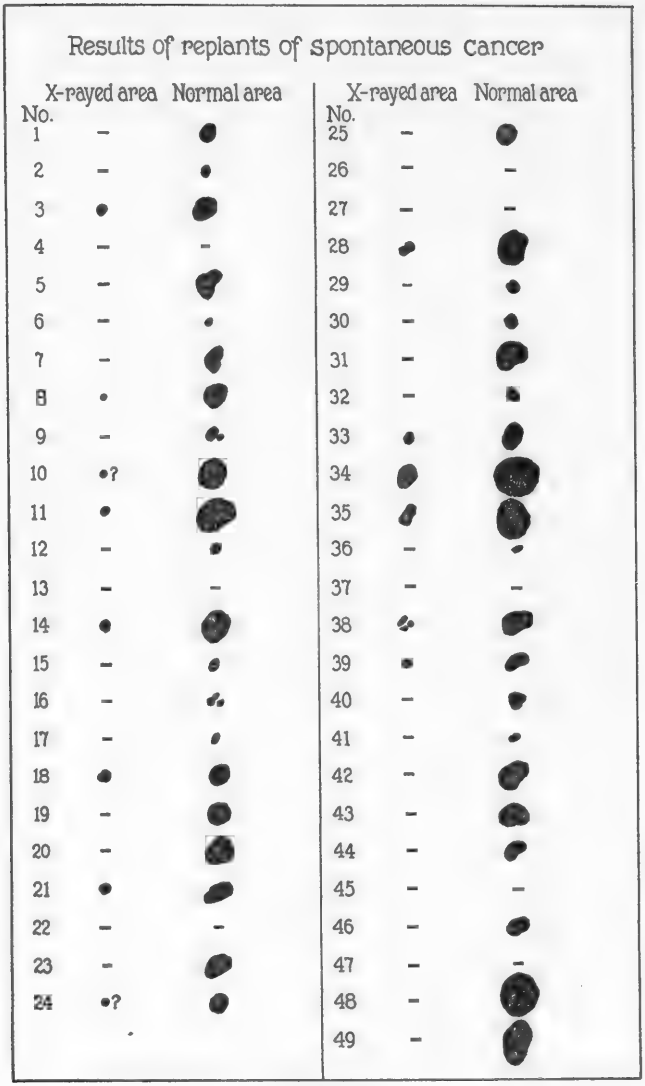
Spontaneous tumor mice

Autografts grew in x-rayed area in 30.6%, in normal area in 83.7%

50 mice so treated



TEXT-FIG. 1.


Results of replants of spontaneous cancer
No.
X-rayed area
Normal area
1 –
2 –
3 •
4 –
5 –
6 –
7 –
8 •
9 –
10 •?
11 •
12 –
13 –
14 •
15 –
16 –
17 –
18 •
19 –
20 –
21 •
22 –
23 –
24 •?
25 –
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33 •
34
35
36 –
37 –
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41 –
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43 –
44 –
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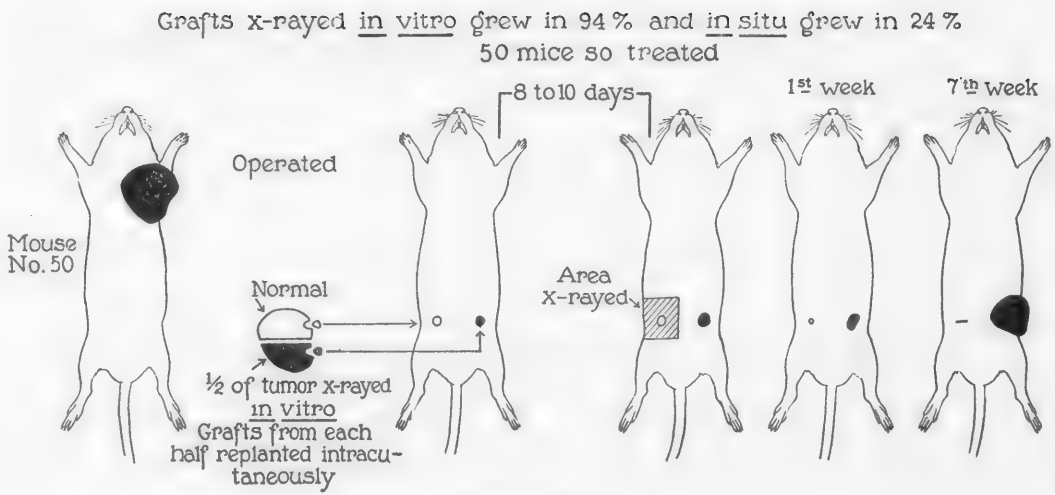
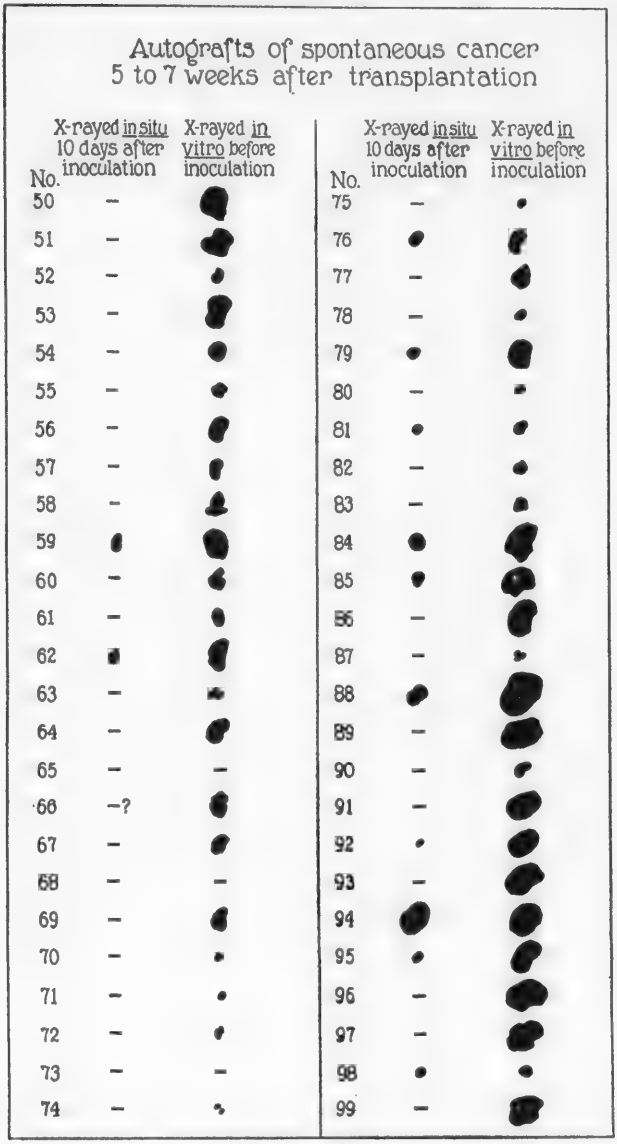
47 –
48 –
49 –

TEXT-FIG. 2.


Grafts x-rayed in vitro grew in 94 % and in situ grew in 24 %
50 mice so treated
8 to 10 days
1st week
7th week
Operated
Mouse
No. 50
Normal
Area
X-rayed
½ of tumor x-rayed
in vitro
Grafts from each
half replanted intracu-
taneously

TEXT-FIG. 3.


Autografts of spontaneous cancer
5 to 7 weeks after transplantation
No.
X-rayed in situ 10 days after inoculation
X-rayed in vitro before inoculation
50 – 51 – 52 – 53 – 54 – 55 – 56 – 57 – 58 – 59 60 – 61 – 62 63 – 64 – 65 – 66 –? 67 – 68 – 69 – 70 – 71 – 72 – 73 – 74 –
75 – 76 77 – 78 – 79 80 – 81 82 – 83 – 84 85 86 – 87 – 88 89 – 90 – 91 – 92 93 – 94 95 96 – 97 – 98 99 –

TEXT-FIG. 4.

amperes 10; distance 6 inches; time 2½ minutes; no filter.) A small bit of this portion of the tumor, taken from the surface nearest the x-ray tube, was inoculated intracutaneously in the right flank of the original animal.[4] A graft of similar size from the untreated portion of the tumor was inoculated in the same way in the left flank (Text-fig. 3).

In the fifty mice subjected to this experiment, the grafts from the cancer x-rayed outside the body, with perhaps two exceptions, grew as rapidly as the untreated cancer, and in many cases more rapidly. After about 10 days, sometimes longer, when the new tumors had become established, the one which originated from the untreated graft was given the same dose of x-rays *in situ*, which the other tumor had received *in vitro*, the treatment including the surrounding normal skin as well as the tumor. This was followed by a prompt disappearance of the tumor in thirty-eight of the fifty animals (76 per cent) so treated, whereas the grafts from the portion of tumor x-rayed *in vitro* continued to grow in forty-seven of the fifty mice, failing in only three (6 per cent). In the twelve instances in which the tumor x-rayed *in situ* did not disappear after treatment, without exception it grew at a slower rate than the other tumor arising from the graft which had been x-rayed *in vitro* (Text-fig. 4).

There seems little doubt from the results of this experiment that a treatment dose of x-rays fails to have any appreciable direct effect on the cancer cells, yet the same dose given to a growing cancer together with the surrounding normal tissue brings about healing in a majority of cases.

There is still another point to be considered. Is it possible that tumors x-rayed *in situ* are more sensitive than those exposed *in vitro?*

Experiment 3.—As in the previous experiments, a spontaneous mouse tumor was removed at operation, and without treatment either to the tumor or the animal, small bits of the tumor were reinoculated intradermally in both flanks. After the resultant tumors were well established and growing actively, one of them was exposed *in situ* to the same dose of x-rays as that used in the preceding experiment. After the treatment this tumor was removed and again reinoculated into an unrayed area of the same animal. Forty-seven mice with spontaneous cancer received this treatment and in thirty-seven (78.8 per cent) instances the x-rayed tumor grew well in its new location.

It would seem therefore that there is no increased susceptibility of tumor cells to x-rays when treated *in situ*, and that tumor treated in

[4] With the quality of x-rays used here the increased dosage due to scattering would be theoretically as great in the locality from which the graft was taken as in a tumor of the surface layers of an animal exposed to the same initial dosage.

such a fashion, when removed from the unfavorable environment induced by the x-rays, will grow actively when replanted in a new location on the same animal.

DISCUSSION.

The fact that a large proportion of certain forms of skin cancer yield to x-ray and radium treatment is one of the chief supports for the belief that the malignant cell is more susceptible to radiation than normal tissue. An attempt has been made in the experiments reported here to analyze the mechanism by which x-rays affect the tumor lying within the skin layers. The extent to which one is justified in assuming similarities between the behavior of tissue in man and lower forms of animals is still a question, but there is little doubt that spontaneous cancer as it occurs in animals closely resembles the disease in man. It is evident from our experiments that, as far as mouse cancer is concerned, the beneficial result from x-ray therapy is due to the reaction in the normal tissues induced by the rays, not to any direct effect on the cancer cells. That this point, first brought out with a transplantable tumor, and now confirmed for the spontaneous disease, may hold true for human cancer is not improbable. Statements by Ewing,[5] based on a careful study of human material, indicate that the reaction induced in the surrounding normal tissues by x-rays or radium is of as great importance as we have shown it to be in animals. In a recent address he makes the following statements. "It is clear that the reaction of the tissues is an essential factor in the curative process. Under some circumstances, when this reaction fails, no amount of radiation succeeds in killing the tumor cells . . . . the most detailed knowledge we possess indicates clearly that the curative action is not the result of a direct effect exclusively upon the tumor cells, but involves especially a peculiar reaction of the normal or invaded tissues."

Whether the beneficial results from the use of high frequency x-rays depend on the same factors is a point as yet undetermined; but this seems not improbable, since the maximum amount of x-rays supposedly delivered to the deep tumors in such treatment is well below the

[5] Ewing, J., *Am. J. Roentgenol.*, 1922, ix, 331.

experimentally established lethal dose for cancer cells. The wave length of the rays used in deep therapy is shorter than that of those previously used, yet longer than the gamma rays of radium. Since both the relatively long x-ray waves and the short waves of the gamma ray in all probability influence cancer through the reaction induced in the normal tissue, it is not unreasonable to expect that the high frequency x-rays will eventually be found to act in the same way.

SUMMARY.

Autografts from spontaneous cancers of mice when replanted into areas previously exposed to an erythema dose of x-rays, failed to grow in the majority of instances (71.4 per cent), while similar grafts inoculated into untreated areas grew in a large proportion of the animals (83.6 per cent).

Autografts of spontaneous cancer, established and growing in the skin, disappeared in 76 per cent of animals after the tumor and surrounding tissues had been exposed to an erythema dose of x-rays, whereas other autografts of similar derivation that had been given a like dose of x-rays outside of the body and had been implanted in the same animals grew progressively in 96 per cent of instances. That this result was not due to a greater susceptibility of the cancer cells x-rayed *in situ* was shown by the fact that tumors treated *in situ* with x-rays and then replanted in an unrayed location on the same animal grew actively. Evidently the ray had done no direct damage to the cancer cells.